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RESEARCH ARTICLE

Evaluation of *Annona muricata* Linn Leaves Extracts from Maceration and Ultrasonic-Assisted Methods for Anticancer Activity on HLFa Cells

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Abstract Non-small cell lung cancer (NSCLC) is a deadly kind of cancer that contributes significantly to the global cancer mortality rate. It is distinguished by a significant level of malignancy and unfavourable prognosis. The molecular pathways responsible for tumour invasion and migration in NSCLC are not fully understood, despite their widespread occurrence and significant consequences. Moreover, the ability of cancer cells to withstand the effects of chemical treatments presents a substantial obstacle in the creation of successful treatment approaches for NSCLC. *Annona muricata* Linn (*A. muricata*) is known to possess powerful anticancer bioactive components. *A. muricata* extracts have demonstrated significant therapeutic potential among a wide range of botanical compounds. However, the specific molecular interactions of the plant have not yet been revealed. This study aims to evaluate the anticancer potential of *A. muricata* leaves extracts on the NSCLC cell line and compare the efficacy of two different extraction methods, namely maceration extraction (ME) and ultrasonic-assisted extraction (UAE). Results showed that ME demonstrated significantly higher antioxidant activity compared to UAE, with respective percentages of 81.4% and 29.4%. However, the UAE extract demonstrated more pronounced cytotoxic effects on the NSCLC cell line (HLFa) with an IC50 value of 139.6 µg/ml, indicating a stronger antiproliferative effect on cancer cell. Both ME and UAE extracts reduced nitrite release in HLFa cell supernatants, with the ME extract showing superior activity. Treatment with ME and UAE also resulted in the activation of Caspase3/7, indicating the induction of apoptosis in HLFa cells compared to the untreated control. The extracts and Cisplatin differ approximately 0.3-fold in caspase 3/7 activation though it was not statistically significant. This activation suggests that both extraction methods effectively initiate the apoptotic cascade which is crucial for the elimination of cancer cells. Furthermore, the UAE extract significantly reduced BCL-2 mRNA levels ($p<0.05$). The significant reduction in BCL-2, a protein that prevents apoptosis reflect the extract' ability to modulate key apoptotic regulators with the most significant activity when UAE extracts were used. In summary, *A.muricata* leaves extracts obtained through both ME and UAE methods exhibited promising anticancer effects against NSCLC, with UAE extracts exhibiting superior activity. These findings pave the way for further investigations into the use of *A.muricata* in cancer treatment and the development of new therapeutic agents based on its properties.

Keywords: *Annona muricata* L., antioxidant, lung cancer, anticancer, non-small cell lung cancer, Soursop, Graviola.

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Introduction

Lung cancer is a malignancy characterised by the highest cancer mortality rate, with an 18% fatality rate, and a low five-year survival rate ranging from 10% to 20%. In 2020, based on data from 185 countries, the approximate number of diagnosed cases was estimated at 2,206,771, accounting for 11.4% of all cancer diagnoses worldwide while mortality was around 1,796,144 deaths, representing 18% of all cancer-related deaths globally. Mortality remains a critical concern, especially in countries like Hungary and China where healthcare resources and air quality improvements vary widely [1]. Non-small cell lung

cancer (NSCLC) accounts for around 80-85% of all lung cancers. The two main types of NSCLC are adenocarcinoma, which makes up about 40-50% of cases, and squamous cell carcinoma, which makes up about 20-30% of cases [2, 3]. NSCLC is a prevalent form of lung cancer, accounting for around 85% of cases [4]. More than 60% of patients diagnosed with NSCLC at advanced stages (stage III and IV) are not eligible for surgical resection. The high incidence of lung cancer poses substantial risks and difficulties to both human well-being and societal and economic progress. Historically, lung cancer patients have primarily relied on traditional chemotherapy and radiation therapy for treatment. Emerging research suggests that certain natural substances have promise as alternative therapeutic options for cancer, particularly lung cancer.

Phytochemicals have been utilised since the beginning of medicine to help humans maintain their health [5]. Phytotherapy, or herbal medicine, has been used to treat a variety of illnesses, including cancer [6]. Dietary phytochemicals possess inherent benefits compared to synthetic molecules, as they have demonstrated safety, affordability, and the ability to be absorbed orally [7]. Recently, researchers have started exploring how plant-derived substances work at the molecular, cellular, and tissue level [8-10]. A wide range of natural products have undergone thorough research, revealing various substances that have demonstrated anticancer and other advantageous effects in contemporary controlled trials. Anticancer natural products hinder the onset, growth, and advancement of cancer by influencing multiple mechanisms such as cell growth, specialisation, cell death, blood vessel formation, and spread to other parts of the body [11]. Embelin, a type of quinonoid, has been found to hinder the progression of lung cancer by activating the MAPK signalling pathway through oxidative stress, as described by Ying *et al*. in 2020 [12]. In addition, Sun *et al*. (2021) investigated the impact of natural substances, specifically rocaglamide and tanshinone IIA, on augmenting the ability of natural killer (NK) cells to eliminate NSCLC cells [13]. These investigations emphasised the capacity of natural products to regulate the immune response against lung cancer.

Annona muricata L., commonly referred to as soursop or graviola, is a tropical tree that produces fruit and belongs to the Annonaceae family. The species is distributed in the rainforests of Africa, South America, and Southeast Asia [14-19]. The plant has attracted considerable attention due to its varied medicinal characteristics and possible therapeutic uses [20]. *A. muricata* has undergone comprehensive research into its traditional medicinal applications, phytochemical makeup, pharmacological properties, and biological impacts. The plant is extensively grown throughout Southeast Asia including Malaysia [21]. The species is also present in the most equatorial parts of Central and South America, Southeast Asia, and Western Africa [16, 17]. *A. muricata* possesses a somewhat acidic flavour when fully mature and is frequently cultivated in Asia, South America, and numerous tropical islands [17]. The wide array of research conducted on *A. muricata* highlights its importance as a reservoir of bioactive substances that may have promising therapeutic uses. *A. muricata*, known for its traditional medicinal purposes, is currently the focus of intensive research due to its pharmacological activities and biological impacts. This study aims to evaluate the anticancer potential of *A.muricata* leaves extracts on the NSCLC cell line and compare the efficacy of two different extraction methods, namely maceration extraction (ME) and ultrasonic-assisted extraction (UAE).

Materials and Methods

Collection and Preparation of Plant Material

The *A. muricata* leaves were freshly harvested from a plant cultivated within the premises of the Institute of Bioproduct Development, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia. Dr. Shamsul Khamis, a botanical specialist from the Institute of Bioscience at Universiti Putra Malaysia in Serdang, Selangor, Malaysia, identified the leaf sample. The leaf sample's voucher specimen number (Reference No.: SK2293/13) was stored in the Herbarium, Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, located in Serdang, Selangor, Malaysia. The leaves were cleansed and purified using distilled water. The leaves were dehydrated in the oven at a temperature of 40 °C until the moisture content of the leaves dropped to less than 10%. The desiccated leaves were ground and stored at ambient temperature in an airtight receptacle for subsequent utilization.

Preparation of the Extracts

The samples were prepared through the application of ultrasonic-assisted extraction (UAE) and maceration (ME) techniques. The parameters for both condition were optimized using response surface methodology (RSM). For ultrasonic-extraction (UAE) process, 3 g of the ground *A. muricata* leaves was weighted and added into 10 mL of ethanol in a tube. Then the sonicator probe was inserted into the extraction mixture. The amplitude was set at 70% for 15 minutes. In the maceration extraction (ME) process, 20 grams of ground leaves were soaked in 100 mL of ethanol and stirred at 200 rpm for 16

hours. Both extracts were then subjected to evaporation using rotary evaporator. Once, the solvent completely evaporated, the extracts were dried in the oven at the temperature of 40 °C. The dried extracts were stored at -20 °C for further analysis.

Free Radical Scavenging Assay

The free radical scavenging activity of the extracts was determined using the DPPH method. Each sample (UAE and ME) was prepared by adding 50 mg of the sample into 1 mL of methanol. An amount of 100 µL of each sample was added into 100 µL of 0.1 mM DPPH reagents, and the mixtures were mixed thoroughly. After 30 min of incubation at room temperature in the dark, the absorbance was measured against a blank of methanol at 515 nm using a visible spectrophotometer. Standard free radical scavenger ascorbic acid (vitamin C) was used as a positive control. Meanwhile, the negative control was prepared with 100 µL of 0.1 mM DPPH reagent and 100 µL of methanol without extracts or standard. At the end of the experiment, the result was expressed in mean average, and the free radical scavenging activity was calculated following the equation given below:

$$\text{Inhibition (\%)} = \frac{Acontrol - Asample}{Acontrol} \times 100$$

Cell Culture

HLFa, an epidermoid carcinoma cell line was a kind gift from Professor Datuk Dr. A Rahman A Jamal, the founding director of UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 100 U/L penicillin, and streptomycin (Gibco Inc), at 37 °C ± 1 °C in a humidified atmosphere of 5% CO2. All cells used were below passage 20. HSF1184, a fibroblast cell line was a kind gift from Assoc Prof. Dr. Rosnani Hasham from the Department of Bioprocess Engineering, Universiti Teknologi Malaysia. The cells were maintained in DMEM (Sigma-Aldrich), supplemented with 10% FBS, 100 U/L penicillin, and streptomycin (Gibco Inc), at 37 °C ± 1 °C in a humidified atmosphere of 5% CO_2. All cells used were below passage 20.

Antiproliferation Study

HLFa and HSF1184 cells were seeded in 96-well plates for 24 h at 37 °C in humidified 5% CO_2 atmosphere prior to treatment. After that, cells were incubated with UAE and ME at various extract concentrations (3.90625, 7.8125, 15.625, 31.25, 62.5, 125, and 250 µg/mL) for treatment purposes. Wells containing DMEM were added for blank. Cisplatin was also used to treat the HLFa cells as a positive control. After 48 h of treatment, cell viability assays, MTT, were performed [22]. An amount of 50 µL of MTT solution (5 mg/mL in PBS) was prepared and subsequently inserted into the treatment wells. Then, the treatment plate was covered in aluminum foil and incubated for 4 h in a humidified incubator. After that, the medium from every well was carefully discarded using an aspirator. The formazan crystals formed at the bottom of the wells were solubilized using 100 µL of DMSO. The mixture was gently stirred. A purplish mixture slowly formed inside the wells. The absorbance of the purplish mixture was recorded with a spectrophotometric microplate reader at 575 nm with 670 as a reference wavelength. The mean absorbance was calculated, and the graph for the percentage of inhibition was constructed following the given formula. Once the graph of cell viability was plotted, IC50 value was determined.

$$\text{Inhibition (\%)} = \frac{Acontrol - Asample}{Acontrol} \times 100$$

Nitric Oxide Determination

To determine the release of nitric oxide after treatment in HLFa, 1×10^5 cells were seeded per well on a 96-well plate in DMEM supplemented with 10% FBS and 1% antibiotics. After overnight incubation, several concentrations (3.906, 7.813, 15.625, 31.25, 62.5, 125, and 250 µg/mL) of the extracts were added into triplicate wells. Then, 48 h later, approximately 50 µL of the supernatant of each sample was transferred into a fresh 96-well plate. Griess Reagent System (Promega, USA) was used to measure nitrite. The Griess reagents were added, 50 µL of sulfanilamide and 50 of µL N-(1-Naphthyl) ethylenediamine dihydrochloride, respectively, following the manufacturer's protocol. After 30 min of incubation at room temperature, the absorbance was read at 540 nm using the ELISA multi-plate reader.

Apoptosis Detection Assay

In this study, apoptosis induced by the extract was determined by measuring the activity of caspase 3 and caspase 7. Caspase-Glo® 3/7 (Promega, G8091), purchased from Promega (Madison, Wisconsin,

USA). HLFa cells were seeded in 96 well plates at a density of 1 × 10^5 cells/ml. Cells were incubated in an incubator for 24 h. After that, cells were treated with the extract, ethanol (vehicle), cisplatin, and blanks. Cells were incubated again for 48 h in the incubator. After 48 h, the treatment plate was removed from the incubator and left to equilibrate at room temperature. Caspase-Glo® 3/7 Reagent was prepared and allowed to equilibrate at room temperature. An amount of 100 µL of Caspase-Glo® 3/7 reagent was inserted into each well. The contents were gently mixed using a plate shaker for 30 seconds. The plate was incubated at room temperature for 1 h. After 1 h, the luminescence of each sample was measured using a luminometer (Promega Luminometer). The assay was performed in triplicate. The results were calculated using the following equation:

$$RLU = Sample - Blank$$

RLU stands for relative light unit. It is a unit for measuring cleanliness by measuring the levels of Adenosine Triphosphate (ATP).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

After exposing HLFa cells (5 × 10^5 cells/well) to UAE extract (50 µg/mL), ME extract (50 µg/mL), and cisplatin (10 nM) for 48 h, total RNA from HLFa was isolated and purified using a Quick-RNATM MiniPrep kit (Zymo Research, Irvine, CA, USA) following the provided manufacturer's protocol. cDNA was synthesized according to the protocol provided in Promega Reverse transcription system A3500 kit (Promega, USA). Table 1 shows the primer used in the amplification. GAPDH was preserved as a reference gene. The assay was performed on CFX96TM real-time PCR system (Biorad Laboratories) in 96-well format and 20 µL reaction volume per well using TaqManTM Fast Advance Mastermix (Foster City, CA, USA).

Table 1. List of primer

Gene	Gene ID
BAX	Hs00180269_m1
BCL2	Hs00608023_m1
GAPDH	Hs99999905_m1

Statistical Analysis

Data are presented as means with their standard errors of means (SEM). Statistical analysis of the data was carried out using the IBM SPSS Statistics Version 24 (IBM Corporation, Armonk, New York, United States). The values of $P < 0.05$ were considered to be statistically significant. All experiments were conducted three times independently in triplicate. Each sample was compared to the control group with IBM SPSS by using the Student's t-test to determine any significant effect of the samples.

Results and Discussion

Antioxidant Properties of *Annona Muricata* Extracts

The antioxidant properties of *A. muricata* have been a focal point in numerous research investigations [23]. In this study, ME exhibited a significantly higher level of antioxidant activity, whereas UAE demonstrated a lower level of antioxidant activity. The experimental value of the antioxidant effect under the ME optimal conditions was 81.4% contrasting with the 29.4% value obtained for UAE under its optimal conditions. Despite these findings, when considering factors such as time, raw materials usage and solvent consumption, UAE emerges as a more favourable technique due to its rapid and efficient nature. Additionally, ultrasonic has been shown to decrease the extraction period and increase extraction yield in various plant materials [24]. The application of UAE has been explored, highlighting the potential of UAE in enhancing the extraction of bioactive components from *A. muricata* [25].

Interestingly our finding diverges from the findings of previous studies that concluded UAE to be more effective in extracting antioxidants compared to ME. Anaya-Esparza *et al*. (2018) conducted a study where they compared ultrasonic-assisted extraction with conventional extraction methods [26]. In their findings, ultrasonic-assisted extraction yielded greater amounts of phenolic compounds and exhibited higher antioxidant activity. Similarly, Xu *et al*. (2019) validated that the utilisation of ultrasonic-assisted extraction resulted in the highest concentration of uronic acid and sulphate, along with the most superior

antioxidant activity in polysaccharides derived from *Amomum villosum* [27]. In addition, Shaharuddin *et al*. (2019) showed that the utilisation of superheated steam drying in conjunction with ultrasonic-assisted extraction resulted in an increase in the overall phenolic content and a boost in antioxidant activity [28].

Table 2. Antioxidant activity of ascorbic acid and *A.muricata* extracts

Sample	Antioxidant activity (%)
Ascorbic acid	100.0
ME extract	81.4
UAE extract	29.4

Antiproliferative Activity of *Annona Muricata* Extracts

Endogenous oxidative DNA damage has been implicated to be a crucial factor in the initiation of cancer. The cancer-protective effect of natural products from plants is attributed to the capability of antioxidative compounds to scavenge free radicals, obstructing DNA damage and ensuing mutation [29]. Due to the possibility of antioxidants diminishing the risk of forming cancer, it is vital to be able to determine antioxidant activity using biologically relevant procedures and assays [30]. MTT was used to determine the antiproliferation capacity of the extracts against HLFa cells. As shown in Figure 1, results show that ME exhibited low antiproliferation activity when compared to UAE. Nevertheless, ME and UAE showed no cytotoxicity against normal fibroblast cells. These findings suggest that ME and UAE inhibited cell proliferation of lung carcinoma cancer cells while showing no cytotoxicity effect on normal HSF1184 fibroblast cells (data not shown) indicating its specificity against cancerous cells. This result is corroborated by several other anticancer studies of *A. muricata* that have demonstrated the potential of *A. muricata* extracts in inhibiting the proliferation of various cancer cell lines. Chikwana and colleagues demonstrated the antiproliferative activity of ethanolic extracts of *A. muricata* leaves, proposing an anticancer mechanism that included the contribution of antioxidant compounds [31]. The study found that the ethanolic fruit extract of *A. muricata* significantly inhibited the growth of RD cells (a type of rhabdomyosarcoma cells) in a concentration and time-dependent manner. Furthermore, the study also revealed that the c-Myc and FGFR1 genes were under-expressed in RD cells treated with the *A. muricata* ethanolic fruit extract. The results also align with another research study that demonstrated the cytotoxic effects of soursop ethanol extract on inhibiting migration of WiDr colon cancer cells [32]. Methanol extracts from the leaves of *A. muricata* demonstrated anticancer effects on two types of cancer cells: Ehrlich Ascites Carcinoma (EAC) and Dalton's Lymphoma Ascites (DLA)1 [33]. The study by de Castro Nascimento *et al* (2019) which involved treating different cell lines (A549, U87, U251, K562, and VERO) with the acetonic or methanolic leaf extracts of *A. muricata* demonstrated that the K562 cell line to be the most sensitive to the treatment with the acetonic and methanolic extracts, with IC_{50} values of 28.82 (24.41 - 34.69) and 32.49 (27.21 - 40.16) µg/mL, respectively. Both extracts were found to induce apoptotic cell death and G0/G1 phase cell cycle arrest [34].

ME demonstrated a notably strong antioxidant effect which will contribute to its effectiveness in inhibiting cell growth. However, the substantial antioxidant activity of ME did not result in a corresponding positive antiproliferation effect. Conversely, a similar pattern was observed with UAE, where despite exhibiting low antioxidant activity, it displayed a potent antiproliferation effect. The IC_{50} of UAE is 139.6 µg/mL, which is lower than 1000 µg/mL. This aligns with previous study which support that an extract exhibits anticancer and antiproliferation activity when the IC_{50} value is less than 1000 µg/mL after 24h [35]. The apparent absence of anticancer activity in ME, despite its notable antioxidant activity, raises several hypotheses regarding the mode of action of potential anticancer substances in *A. muricata*. This discrepancy has been attributed to the absence of specific compounds in certain extraction methods, which may contribute to the contrasting anticancer activity observed in different studies [36]. The presence of a diverse array of bioactive compounds in *A. muricata*, such as alkaloids, flavonoids, terpenoids, and acetogenins, has been well-documented in the literature [36-38]. These compounds have been associated with various bioactivities, including immunomodulatory, anti-inflammatory, anticancer, antiparasitic, insecticidal, antimicrobial, and antioxidant properties [39]. Methanolic fraction of leaves ethanolic extracts demonstrated a high total acetogenins content and total polyphenols content [40]. This findings indicate the capability of methanol to extract the bioactive components that is responsible for most of the plant pharmacological effects.

Furthermore, the specific bioactive constituents responsible for the major anticancer, antioxidant, anti-inflammatory, and antimicrobial benefits of *A. muricata* have been identified, highlighting the multifaceted nature of its bioactive components [14]. The complexity of *A. muricata*'s bioactive profile and its potential variations in different extracts have prompted the need for further studies to elucidate the reasons for

these dissimilarities [36]. The identification and characterization of bioactive compounds in *A. muricata* extracts, particularly those responsible for its anticancer properties, remain crucial for understanding its therapeutic potential. Additionally, the use of advanced analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS) and metabolomics, has been proposed to evaluate the bioactive compounds contained in *A. muricata* extracts and to determine the putative compounds responsible for their bioactivity [38].

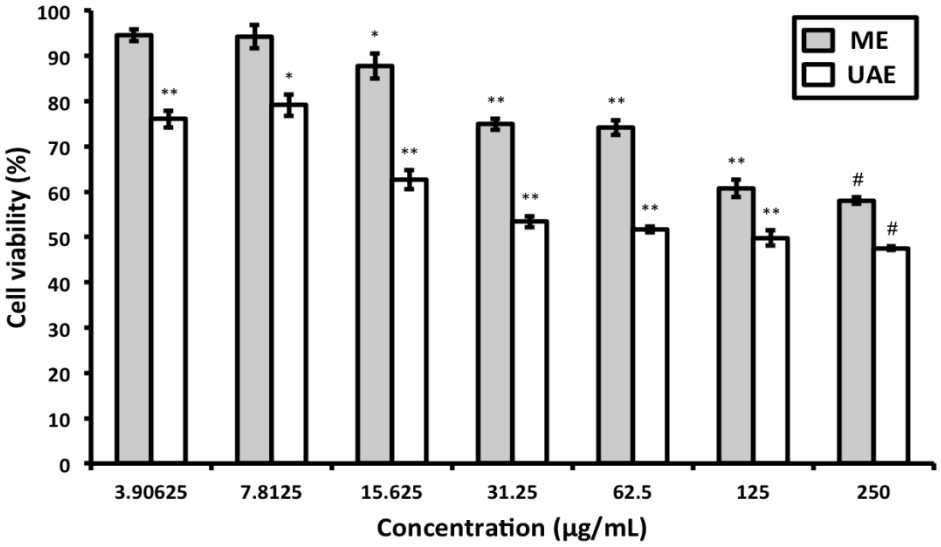

Figure 1. HLFa cancer cells viability after 48 h treatment with UAE and ME at different concentrations. Data are mean ± SEM; n = 3 experiments. *P < 0.05, **P < 0.01, #P < 0.001 against control

Effect of the Extracts on NO Production

Nitric oxide, NO, a free radical generated from L-arginine, is a pro-inflammatory mediator involved in several physiological occurrences, and it is particularly vital to body's defence mechanism. Nevertheless, its overproduction can induce tissue damage and activate pro-inflammatory mediators linked with acute and chronic inflammation [41]. NO, a product of NOS which is highly expressed in cancer cells, also has been implicated to involve in the carcinogenesis and closely related to progression of cancer [42-45]. Continuous expression of this mutagenic NO could lead to tumour growth, angiogenesis and metastasis [46, 47]. Therefore, by measuring the capacity of the extracts to inhibit NO production, suggests the potential presence of anticancer properties. Griess reagent assay was used to measure the NO produced after the treatment with the extracts. As shown in Figure 2, nitrite concentration decreased significantly ($P < 0.1$) after being treated with ME. The results also show a decrease in nitrite concentration following the treatment with UAE; however, with no statistical significance. As compared to the UAE, ME showed a tremendous decrease in NO production. The results for both ME and UAE demonstrated slight variety among various concentrations. This might be caused by the degradation of the nitrite or the possibility of the assay overestimating the presence of nitrite and/or nitrite release [48]. Furthermore, it might also be caused by the short-lived nature of NO [45]. Nevertheless, this result indicates the potential of *A. muricata* extracts to inhibit the production of NO in HLFa cancer cells.

Investigation of plant extracts' effect against NO production is nothing new. To date, there were numerous studies reported findings on this subject of interest. Seventeen commonly used Indian medicinal plants showed potent and novel NO scavenging activity, thus suggesting their therapeutic properties [49]. A study on *Sarcocephalus pobeguinii* had found that the leaves extract of the plant demonstrated inhibitory activity on NO production using RAW 264.7 macrophage cells model [50]. On the other hand, *Fraxinus micrantha* methanolic extract was found to be inducing the production of NO in a concentration and time dependent-manner [51].

The inhibition of NO is a crucial aspect of anti-inflammatory activity. Inflammation is a well-established factor in the development and progression of cancer [52]. The role of NO in cancer is also significant, as it has been linked to various aspects of cancer development and progression [53]. The potential of *A. muricata* extracts to modulate NO production is particularly relevant in the context of cancer, as NO has been implicated in cancer progression and metastasis [54]. Several studies have investigated the

potential of *A. muricata* extracts in inhibiting NO production. However, only a few carried out to investigate the capacity of *A. muricata* extracts on NO production in cancer. Level of NO displayed a lower level in 4 T1 tumor after treatment with *A. muricata* leaves extract as compared to the control group [55]. A study by Kim *et al*. (2016) reported that *A. muricata* leaves extract upregulated the expression of inducible NOS thus leading to the increased production of NO [56]. Additionally, *A. muricata* leaf extracts exhibited inhibitory effects against multidrug-resistant Salmonella, indicating its potential in modulating NO production [57]. These findings suggest that *A. muricata* extracts have the potential to inhibit NO production, which is a key factor in the anti-inflammatory properties of the plant.

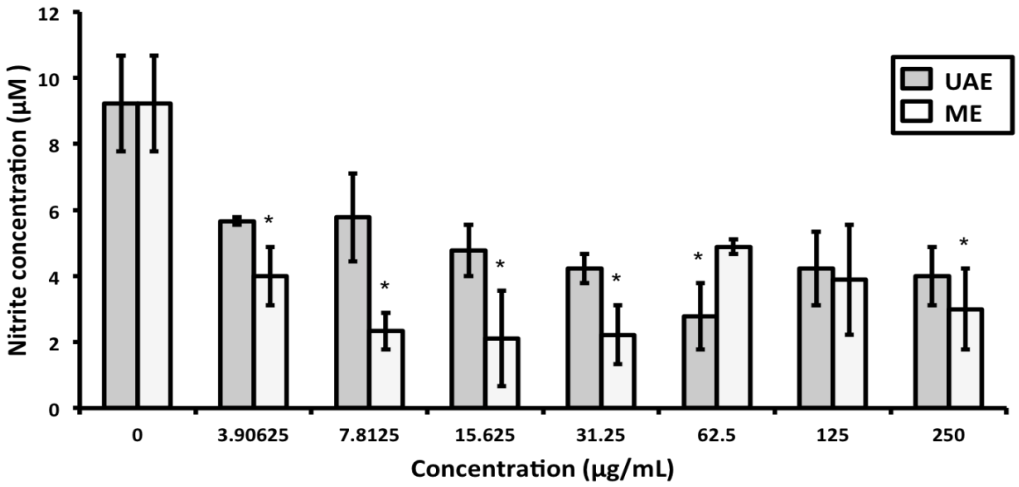

Figure 2. NO concentration in HLFa cancer cells after 48 h treatment with UAE and ME at different concentrations. Data are mean ± SEM; n = 3 experiments. *P < 0.1 against control (0 µg/mL)

Effect of the Extracts on Caspase Activities

As shown in Figure 3, caspase 3/7 was activated after exposure to the extracts (ME and UAE) at 50 µg/mL and 100 µg/mL concentrations after 48h of treatment. The results were compared to the result of cisplatin, which produced significant activation of caspase 3/7 at concentration of 10 nM after 48 h of treatment. These results suggest that the UAE-induced and ME-induced apoptosis happens via the involvement of caspase 3/7 activation. The extracts and Cisplatin differ approximately 0.3 fold in caspase 3/7 activation though it was not statistically significant. However, the results also reveal that for both concentrations of the extracts, the effects are not significantly different. The possible justification for this circumstance is that the apoptosis triggered by the extracts is not solely through the activation of caspases cascade. The cell death might be induced through several mechanisms that synergistically work together. Furthermore, the results also show that caspase 3/7 are readily activated in untreated HLFa cells. A possible clarification for this condition is that caspases also play roles in prosurvival of the cells. Thus, their inactivation is counterproductive for the cancer progression [66, 67]. Another possibility is involving the observations made with caspase inhibitors like zVAD-fmk [68]. zVAD-fmk, a peptidic inhibitor, potently blocks caspase activation thus causes death in short-term procedures. However, in longer-term settings, caspases inhibition basically transforms death morphology from apoptosis to necrosis [65].

Caspase-3 and caspase-7 play a crucial role in apoptosis and are necessary for carrying out apoptosis in various cellular situations [58]. The caspases have a role in breaking down various cellular substances, resulting in the distinct structural and chemical alterations linked to apoptosis [58]. Moreover, the essential role of caspase-3 and caspase-7 in triggering programmed cell death (apoptosis) has been demonstrated in diverse cell populations, including cancer cells [59]. Caspase-3 and caspase-7, apart from their specific functions, have been discovered to interact with many signalling pathways and molecules to control apoptosis. The activation of caspase-3 and caspase-7 has been associated with the regulation of the mitochondrial pathway of apoptosis. This mechanism involves the release of cytochrome c and the subsequent activation of caspase-9, finally resulting in the execution of apoptosis [60]. Furthermore, studies have shown that caspase-3 and caspase-7 are involved in controlling the death receptor system. They have crucial functions in facilitating apoptosis triggered by death receptor signalling [61]. Moreover, multiple investigations have emphasised the interaction between caspase-3

and caspase-7, revealing their collaborative impact on facilitating apoptosis [62]. These caspases have been demonstrated to work together to cleave the majority of caspase substrates, highlighting their cooperative function in the implementation of apoptosis [63]. Furthermore, the participation of caspase-3 and caspase-7 in the process of programmed cell death has been proven in many disease scenarios, such as cancer. Research has demonstrated that the initiation of caspase-3 and caspase-7 is linked to the triggering of programmed cell death in cancer cells, underscoring their potential as targets for cancer therapy [64]. Ultimately, the vast amount of evidence confirms the crucial involvement of caspase-3 and caspase-7 in facilitating programmed cell death via several pathways and in diverse cellular environments. Their initiation and interaction with other molecules and pathways emphasize their importance as crucial regulators of apoptosis. To determine whether the extract-induced apoptosis in HLFa cells is mediated through the caspase cascade, it is essential to investigate the activities of executioner caspase 3/7 using a bioluminescent assay. The intensity of the luminescent reading is proportional to the activation of caspases [65].

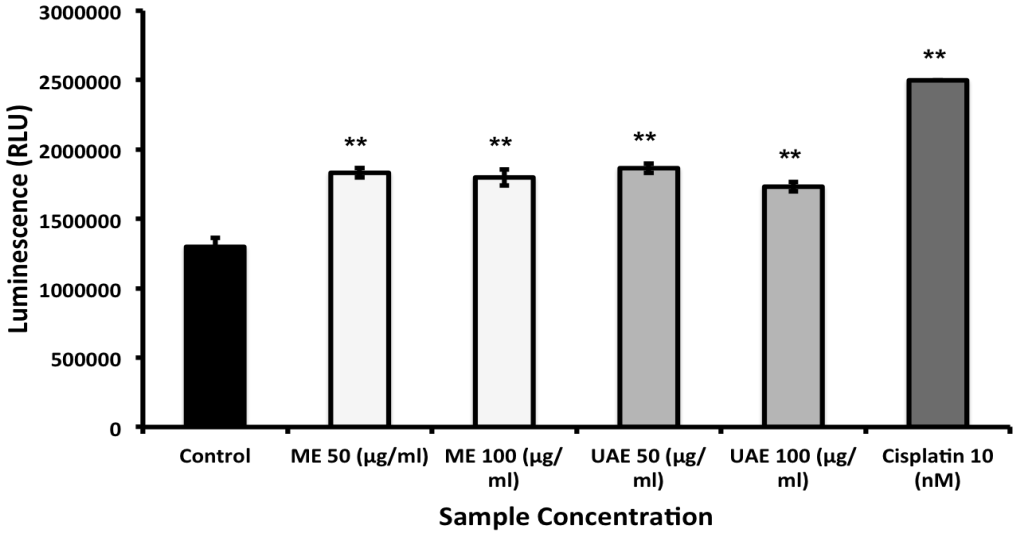

Figure 3. Caspase 3/7 activity in HLFa cells after treatment with the extracts for 48 h. Data are mean ± SEM; n = 3 experiments. **P < 0.01 against control

Effect of the Extracts on BAX and BCL2 mRNA Expression

The mitochondria-initiated events are governed by the regulation of Bcl-2 family of proteins. Progression and termination of this mechanism are controlled by 25 genes in these proteins [69]. Bax, for example, participate in the release of proapoptotic mediators such as cytochrome c, AIF and EndoG to cytosol through protein dimerization and translocation to the outer mitochondrial membrane [70]. The release of these important apoptotic regulators is resulting from the formation of MOMP, a process where mitochondria outer membrane loses its integrity. MOMP in turn activates the apoptotic cascade. The proapoptotic mediators released then activate the formation for apoptosome of which eventually trigger the activation of caspases cascade. As discussed earlier, there was an involvement of executioner caspase 3/7 in the cell death induced by both UAE and ME. To further elucidate the mechanism of action, the levels of both BCL2 and BAX expression were estimated by using quantitative PCR analysis.

In cancer cells, Bcl-2 antiapoptotic proteins such as Bcl-2 and Bcl-xL are highly up-regulated, making these proteins potential target for inhibitory mechanism. For apoptosis to occur through intrinsic mitochondrial pathway, an increase in BAX expression and a decrease in BCL2 expression are typically required. However, in this study, BAX expression was found to decrease significantly following treatment with both ME and UAE extracts. As illustrated in Figure 4, the mRNA expression of the pro-apoptotic gene BAX significantly decreased to half its original level ($p<0.01$) after 48h of treatment with 50 µg/ml of the UAE extract. For BCL2 expression, the UAE extract significantly reduced BCL2 mRNA levels ($p<0.05$) whereas ME extract also led to a decrease in BCL2 expression, but the reduction was not statistically significant.

These findings indicate that the cell death induced by the both UAE and ME was through the activation of caspase-3/7. However, since BAX was down-regulated, the cell death induced might not be via the

activation of caspase-9 and intrinsic pathway. The only possible explanation for this is that the cell death occurred through extrinsic pathways which directly activate caspase-3/7 (Figure 5).

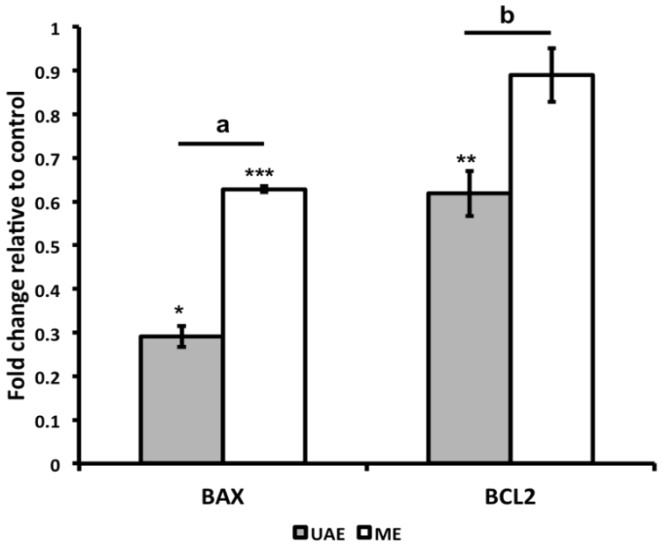

Figure 4. BAX and BCL2 mRNA gene expressions in HLFa cells after 48 h treatment with 50 µg/mL of UAE and ME. Data are mean ± SEM; n = 3 experiments. *P < 0.01, **P < 0.05, ***P < 0.001 against control. (a) P < 0.01, (b) P < 0.05

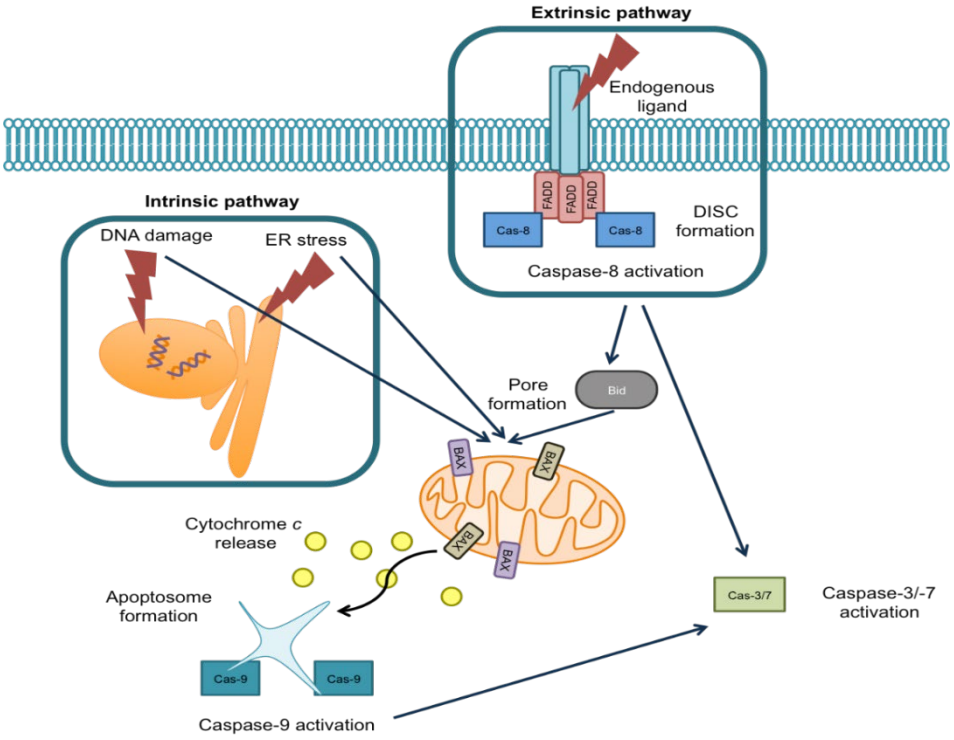

Figure 5. Caspase-3 activation via the intrinsic and extrinsic apoptotic pathways

Conclusions

In conclusion, the extraction of *Annona muricata* using maceration (ME) and ultrasonic-assisted extraction (UAE) has shown promising anticancer activity against non-small cell lung cancer (NSCLC). This study provided comprehensive evidence that these extracts significantly inhibit cancer cell growth, as demonstrated by their impact on cell viability, induction of apoptosis, and reduction in cell proliferation. These findings highlight the potential of *A. muricata* extracts as effective agents in NSCLC treatment. However, further detailed mechanistic studies are essential to elucidate the specific pathways and modes of action through which these extracts exert their anticancer effects. These findings pave the way for further investigations into the use of *A.muricata* in cancer treatment and the development of new therapeutic agents based on its properties.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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