

LC-HRMS profiling and *in vitro* total phenolics, total flavonoids, antioxidant and cytotoxicity evaluation on MCF7 breast cancer cell lines of the extracts and fractions of *Aerva sanguinolenta* from Java, Indonesia

Armansyah Maulana Harahap^{1,2} , Kurnia Agustini² , Nuralih Nuralih² , Sri Ningsih² , Yuli Widyastuti² , Anton Bahtiar^{1*} 

¹Faculty of Pharmacy Universitas Indonesia, Depok 16424, West Java, Indonesia.

²Research Center for Pharmaceutical Ingredient and Traditional Medicine, National Research and Innovation Agency (BRIN), Cibinong Science Center, Bogor, Indonesia.

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ABSTRACT

Aerva sanguinolenta (AS), also called Sambang colok in Indonesian, is a historically utilized herbal treatment in Tawangmangu, Central Java, Indonesia. The herb is claimed to regulate menstrual cycles, inhibit tumor growth, and reduce fever. This study aimed to characterize the phytochemical profile of AS via Liquid Chromatography High-Resolution Mass Spectrometry and to clarify its lethal effects on MCF-7 breast cancer cell lines. The study measured the AS herbal extracts' antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC). Solvent extracts (methanol, ethanol, butanol, n-hexane, and ethyl acetate) and liquid-liquid fractions (n-hexane, ethyl acetate, butanol, and water) obtained from methanol extracts were examined. 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide studies demonstrated considerable cytotoxicity in the n-hexane fraction (IC₅₀ = 49 µg/ml) and ethyl acetate extract (IC₅₀ = 62 µg/ml) against MCF-7 cell lines. Phytochemical investigations revealed the presence of α-Linolenic acid, Galaxolidone, Pinoembrin, and Afzelin in the n-hexane fraction. Extracts demonstrated differing antioxidant (35.53 µg/ml), TFC (14.96 mg QE/g), and TPC (25.61 mg GAE/g) values. In comparison, n-hexane fractions produced unique antioxidant (42.4 µg/ml), TFC [14.96 mg quercetin equivalent (QE)/g], and TPC [25.61 mg gallic acid equivalent (GAE)/g] profiles. N-hexane and ethyl acetate are potential test materials based on cytotoxic test screening against the MCF-7 cell line. N-hexane is the best test material based on all tests conducted, so it becomes the test material that is continued for phytochemical investigations.

INTRODUCTION

The 2022 Global Burden of Cancer Study indicates that breast cancer is the most often diagnosed cancer worldwide, with 2,296,840 new cases (11.7% of all cancer cases), and is the second most significant cause of cancer-related mortality, resulting in 684,996 deaths [1]. Breast cancer is the most common cancer diagnosis in Indonesia [2]. The cancer prevalence rate is roughly 1.4 per 100 individuals, amounting

to 347,000 cases, with cervical and breast cancer demonstrating the most significant incidence rates [3,4]. Research conducted by the American Society of Clinical Oncology analyzed data and samples from 2,062 patients, revealing that the significant subtype of breast cancer was hormone-dependent estrogen receptor (ER+/progesterone receptor+/human epidermal growth factor receptor2-) [5,6].

Prior studies have shown that increased molecular expression of Michigan Cancer Foundation (MCF)-7 is essential in breast cancer, primarily through mechanisms associated with ERs [7,8]. MCF-7 cells exhibit active ERs that, when over-expressed, can enhance cellular responses to estrogen as a growth factor. It promotes cellular proliferation and cancer by activating signaling

*Corresponding Author

Anton Bahtiar, Faculty of Pharmacy Universitas Indonesia, Depok 16424, West Java, Indonesia. E-mail: anton.bahtiar@farmasi.ui.ac.id

pathways such as PI3K/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase, which regulate the cell cycle and their transition from the G1 phase to the S phase [9,10]. Furthermore, increased expression of transcription factors such as c-Myc and Cyclin D1 proximates aberrant cell division [11].

Current research indicates that of the 250,000 plant species in the Plantae kingdom, only 10% have been studied for their anti-cancer potential [12,13]. Many plants are yet unexamined and insufficiently researched for their potential as anti-cancer agents despite their ethno-pharmacological uses as anticancer medications, menstruation stimulants, and birthing facilitators. This is a substantial possibility for identifying anti-cancer chemicals from herbal sources [14]. *Amaranthaceae* is a medicinal plant with several pharmacological properties, including pro-apoptotic, antioxidant, and anti-inflammatory [15]. Breast cancer is a multifactorial disease with complex molecular mechanisms involving ER [16]. *Aerva Sanguinolenta* (AS), a member of their *Amaranthaceae* family, widely known as Sambang Colok (SC), comprises polyphenolic compounds, flavonoids, and tannins acknowledged for their natural antioxidant properties [17]. Researchers can assess these anti-proliferative effects and mechanisms of action or plant-extracted substances in breast cancer cells using vitriol methods [18].

The AS plant is empirically utilized as a cancer preventative, to aid menstruation, and as a therapy for fever. AS exhibits pharmacological activity such as anti-inflammatory, anti-cancer, analgesic, anti-diabetic, and antipyretic effects [17–20]. The high antioxidant activity of AS can aid in preserving homeostasis against free radicals and oxidative stress, which can harm DNA, proteins, and cell lipids, critical elements in cancer progression [21]. Numerous studies suggest that antioxidant chemicals can impede the development of cancer cells and trigger apoptosis [22]. Antioxidant compounds such as flavonoids and polyphenols can regulate apoptosis signaling pathways, including the intrinsic pathway involving mitochondria and the extrinsic pathway involving death receptors [23]. Compounds found in red wine can increase the expression of pro-apoptotic genes, such as Bax, and reduce the expression of anti-apoptotic genes, such as Bcl-2, which contribute to the death of cancer cells [24]. Vitamin C can induce excessive oxidative stress in cancer cells, triggering the apoptosis pathway through caspase activation and producing reactive oxygen species [25].

This study will determine SC efficacy (AS) as an anticancer drug via cytotoxicity assays on MCF-7 breast cancer cell lines. AS was extracted utilizing many solvents, including methanol, ethanol, ethyl acetate, hexane, and butanol. We also fractionated their methanol extract using ethyl acetate, n-hexane, and butanol. All extracts and fractions were subjected to assays. The most active extract/fraction was further tested via Liquid Chromatography High-Resolution Mass Spectrophotometry analysis (LC-HRMS) to determine their bioactive components. We also examine the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC). MCF-7 is a cell line originating from human breast carcinoma, exhibiting threats representative of prevalent breast cancer subtypes, including sensitivity to estrogen hormones [26]. This enables researchers to assess the impact of anticancer agents in hormone receptor-positive cells, which is crucial for advancing hormonal therapy.

Furthermore, MCF-7 offers replication and laboratory upkeep benefits, facilitating repeated study and results validation [27]. Previous research has shown that MCF-7 is highly responsive to various anticancer compounds from natural and synthetic sources, making it an effective tool for early screening in discovering new drugs [28]. Through this cell model, researchers can gain insights into the mechanisms of actions of compounds and the potential side effects that may occur, which is critical for developing safer and more effective therapies [6].

MATERIAL AND METHODS

Sample preparation, extraction, and fractionation of AS

AS, known as SC (AS), was harvested from Tawangmangu, Central Java, Indonesia. AS was recognized at their School of Life Science and Technology, ITB Indonesia, under certificate 7881/IT1.C11.2/TA.00/2023. One kilogram of AS plants is cleaned of dirt and dust, dried in an oven (Memmert, Germany) at 40°C–50°C for 24 hours, then ground using a blender (Moulinex, France) until it becomes a fine powder. Next, the extraction is carried out using the maceration method. As much as 500 g of plant powder was placed into the maceration device, and 1 l of technical ethanol (Merck, Germany) was added. The mixture from the maceration is stirred using a magnetic stirrer. After soaking, the mixture is filtered using filter paper to separate the residue from the resulting extract. The filtered extract is then placed into a rotary vacuum evaporator (Büchi, Switzerland) to evaporate the solvent at 40°C and a pressure of 300 mBar, resulting in a thick extract. After the extraction process, the next step is fractionating the obtained methanol extract. As much as 500 g of methanol extract was placed into the fractionation separator (Perekan, Germany). To separate the compounds based on polarity, 200 ml of n-hexane (Merck, Germany) was added to the methanol extract. The mixture was stirred for 10 minutes and allowed to sit for 30 minutes to ensure the fraction separation occurred. After the separation phase, the n-hexane fraction in the upper layer is taken and stored in a storage bottle. The process is done by adding 200 ml of ethyl acetate (Merck, Germany) to the remaining methanol extract, stirring for 10 minutes, and letting it sit for 30 minutes before collecting the ethyl acetate fraction. Next, 200 ml of butanol (Merck, Germany) will be added, and the same process will be performed to obtain the butanol fraction. Finally, the remaining extract is processed by adding 200 ml of water, stirring, and separating it into the aqueous fraction. All the resulting fractions were then evaporated using a rotary vacuum evaporator at the same temperature and pressure to remove the solvent, yielding n-hexane, ethyl acetate, butanol, and water fractions. These fractions are stored in amber glass bottles to maintain stability and quality.

Antioxidant activity: 2,2-dipheynyl-1-picrylhydrazyl free radical scavenging assay

The antioxidant activity was assessed by evaluating the free radical scavenging capacity of their AS extract and fraction against their stable 2,2-dipheynyl-1-picrylhydrazyl (DPPH) free radical, following the method established by [16,29] Sarkar *et al.* [29] and Gulcin and Alwasel [30] with minor changes.

500 mg of their samples and 5 ml of their methanol reaction mixture were combined, vortexed, and sonicated to create a stock solution. Sixteen microliters of each sample were mixed with 1,584 μ l of methanol, followed by the addition of 400 μ l of DPPH, and their mixture was vortexed. The test samples were generated in triplicate at five concentrations ranging from 62.5 to 1,000 ppm, with their maximum concentration being 1,000 ppm (80 μ l + 79,20 μ l of methanol). The solution was incubated at an ambient temperature for 30 minutes before measuring their absorbance at a wavelength of 517 nm with a microplate reader (FLUOstar omega). The quantities of several extracts were quantified, and their IC50 value (their concentration necessary to block 50% of DPPH radicals) was established. All assays were conducted in triplicate. Aquadeis served as controls. Meithanoil, combined with DPPH, served as their control. L-(+)-ascorbic acid (Vitamin C) with a final concentration of 0–5 μ g/ml was used as the standard antioxidant, measured absorbance value was transformed into the percentage of radical scavenging activity utilizing their subsequent equation [31,32]

$$\text{Radical trapping activity (\%)} = \left[\frac{(AB - AS)}{AB} \right] \times 100\%$$

The percentage of radical trapping activity can be determined using their subsequent equation: AB denotes their sample absorption, while AS signifies their absorption of the Blank.

Total flavonoid content

The AlCl₃ approach, predicated on the complexation of AlCl₃ with flavonoids exhibiting a peak absorbance at 415 nm, was employed to quantify the TFC [33,34]. In summary, 60 μ l of ethanol, 5 μ l of 10% AlCl₃, and 20 μ l of each extract (0.5 mg/ml) were mixed separately. After adding 110 μ l of distilled water and 5 μ l of 1 M potassium acetate each week, their reaction mixture was incubated for 25 minutes. To guarantee experimental repeatability, each measurement was conducted in triplicate. A quercetin calibration curve was employed to determine their TFC; the absorbance was measured at 510 nm. The TFC results are expressed as milligrams of quercetin equivalent (QE) per gram of dry extract weight (mg QE/g) via generating a standard curve with a series of concentrations of 0–100 μ g/ml of rutin (x-axis) against absorbance (y-axis).

Total phenolic content

The Folin-Coicalteu method, with slight modifications, was employed to quantify their TPC as described by Cacique *et al.* [35]. In a 96-well microtiter plate, 20 μ l of their sample (0.5 mg/ml) of plant extracts, our standard, and 100 μ l of Folin-Coicalteu reagent (1:10; v/v) were added, and their initial absorbance was recorded. The combination was subsequently combined with 80 μ l of 1 M Na₂CO₃ solution to achieve a final volume of 200 μ l, followed by a 25-minute incubation period. The Synergy LX Multi-Moidei Reader with Gein5 3.08.01 software recorded absorbance at 765 nm. A gallic acid calibration curve was employed to determine their TPC; a standard solution of gallic acid at 1,000 ppm was prepared by dissolving 10 mg of gallic acid in 10 ml of methanol, and a series of standard solutions with concentrations of 300, 250, 200, 150,

and 100 ppm were made, results expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract weight (mg GAE/g). Each measurement was conducted in triplicate for validation purposes.

Cell culture

The MCF-7 cell line, derived from human breast cancer, was procured from the Laboratory for Agro and Industrial Biomedical Technology Development at the National Research and Innovation Agency (BRIN) in Serpong, Tangerang Selatan, Indonesia. It is utilized in the research. MCF-7 cells cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Life Technologies) media contain 10% fetal bovine serum (FBS), red phenol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. The cells are maintained in a 75 cm² container at 37°C, with 5% CO₂ and 95% humidity.

Cytotoxic assay in vitro

The cytotoxicity experiment of MCF-7 breast cancer cells was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) techniques. MCF-7 breast cancer cells were cultured in 96-well plates for 24 hours. A 96-well plate has 1 $\times$ 10⁴ cells pair well, utilizing RPMI media with phenol Red, supplemented with 10% FBS, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and one mM sodium pyruvate. The cells were incubated for 24 hours at 37°C, 5% CO₂, and in a humidified atmosphere at 95%. Subsequently, their media was altered with samples in their growth medium at six varying concentrations and incubated for 24 hours under the same conditions. The salt wash is performed utilizing a phosphate saline buffer. The incorporation of MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium) into their medium was succeeded by a 4-hour incubation at 37°C, 5% CO₂, and 95% humidity to ascertain viable cells. The color intensity is quantified using an ELISA reader at 570 nm, using an EnSpire™ 2,300 multilabel plate reader manufactured by Perkin Elmer in the United States. The formula used to calculate the percentage of inhibition was $(A_{\text{treatment}}/A_{\text{control cell}}) \times 100$, where $A_{\text{treatment}}$ represents the absorbance of the treatment and $A_{\text{control cell}}$ represents the absorbance of the control cell. Data are evaluated by probit analysis to determine IC50 [36,37].

Liquid chromatography high-resolution mass spectrophotometry (HRMS) analysis

This analysis utilized liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) in conjunction with Orbitrap HRMS (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ HRMS). The liquid chromatography employed a Thermo Scientific™ Accucore™ Phenyl-Hexyl analytical column measuring 100 $\times$ 2.1 mm ID $\times$ 2.6 μ m. The methodology employed was derived from [38] utilizing the mobile phase of MS-grades water with 0.1% formic acid (A) and mass spectrometry (MS)-grades methanol with 0.1% formic acid (B), implemented via a gradient approach at a flow rate of 0.3 ml/min. The gradient commenced at 5% mobile phase B, progressively escalated to 90% over 16 minutes, maintained at 90% for 4 minutes, and thereafter reverted

to their initial condition of 5% B by their 25-minute mark. The column temperature was sustained at 40°C, with an injection volume of 3 µl. The non-target screening was performed in a complete MS/dd-MS2 acquisition model with either positive or negative polarity or ionization state. The arbitrary units for nitrogen were 32, 8, and 4, and it served as a sheath, auxiliary, and purge gas. The auxiliary gas heater temperature was set to 30°C, the capillary temperature to 320°C, and the spray voltage to 3.30 kV. The resolution was 70,000 for full MS and 17,500 for dd-MS2 in both positive and negative ionization models, with a scanning range of 66.7 to 1000 m/z. The device was controlled using XCalibur 4.4 software from Thermo Scientific, Bremen, Germany. To ensure optimal instrument performance and robustness in mass accuracy investigations, their device was calibrated weekly in positive and negative ESI models utilizing Thermo Scientific Perce ESI join calibration solution (Waltham, MA).

Data analysis

All experiments were conducted in triplicate. Data are presented as mean values with a $\pm$ SD. Using the GraphPad prism and one-way analysis of variance (ANOVA), We identified significant results using a *p*-value of less than 0.05 using IBMSPSS Statistics 22 (ANOVA) software. All values are represented by (*).

RESULTS AND DISCUSSIONS

Antioxidant activity, TFC, and TPC against AS

The antioxidant efficacy of their extract and fraction was assessed utilizing the DPPH assay. Figure 1 illustrates the effectiveness of AS extract, produced with different solvents, in neutralizing DPPH free radicals compared to gallic acid across multiple concentrations ranging from 31.25 to 500 µg/ml. The DPPH scavenging activity of these different extracts is contingent upon concentration, augmenting with higher extract concentrations. The AS N-Hexane extract and fraction from each tested plant had the best antioxidant activity among the extracts analyzed.

Table 1 shows that the N-Hexane extract and fraction exhibit the best test parameter value compared to their antioxidant extracts and fractions, with the extract value (Antioxidant: 35.53 µg/ml, TFC: 14.96 mg QE/g, TPC: 25.61 mg GAE/g) and fraction values. (Antioxidant: 42.4 µg/ml, TFC: 15.51 mg QE/g, TPC: 25.31 mg GAE/g).

Furthermore, we established a Pearson correlation among TPC, TFC, and the antioxidant activity of their test plants to ascertain the relationship between these biocompositions and their plants' antioxidant activity (Table 2). A very positive association was identified between TFC and the antioxidant activity of the AS plant. The positive correlation suggests that the elevated antioxidant activity of the AS plant extract is attributable to a greater concentration of TPC. The AS fraction exhibits a favorable association and a robust link between antioxidant activity and TFC. Our findings corroborate earlier research indicating a strong correlation between TFC activity and antioxidant activity, as TFC is a prominent antioxidant agent in numerous medicinal plants.

The research results show that the antioxidant test, TPC, and TFC in the n-hexane and ethyl acetate extracts of AS have good levels, with a very high Pearson correlation value between TFC and antioxidant activity, namely 0.900 for the extract and 0.898 for the fraction. This correlation indicates that flavonoid compounds significantly contribute to the antioxidant potential of AS, which is in line with various recent studies affirming the role of flavonoids as cellular protective agents with the ability to capture free radicals and reduce oxidative stress [39]. According to the latest journal, flavonoids not only function as antioxidants but also possess anti-inflammatory activity and can contribute to the prevention of degenerative diseases, such as cancer and heart disease. TPC, which includes other phenolic compounds, also enhances antioxidant activity, indicating synergy between flavonoids and phenolic compounds in the extract [39]. With its high levels of TFC and TPC, AS has the potential to be an effective natural source in traditional and modern medicine, supporting disease prevention strategies through increased intake of bioactive compounds [40]. Table 3 shows that AS extracts have potential as anti-cancer based on the IC50 values produced by N-Hexane and Ethyl acetate extracts have potential, with strong values shown by ethyl acetate extracts and followed by medium N hexane, and for methanol and ethanol extracts have no potential at all.

Cytotoxic activity based on MCF-7 cell viability

Cytotoxic assay results indicate a decline in their viability curves if MCF-7 cells beginning at five concentrations (31.25, 62.5, 125, 250, 500, and 1,000 µg/ml), which visibly diminished their growth curve. This interpretation of viability, based on three repetitions, is illustrated in Figure 3. N-hexane and ethyl acetate extracts demonstrated doses that inhibited 50% of MCF7 cell growth, ranging from 62.5 to 1,000 µg/ml. The IC50 values for each extract are calculated to ascertain the specific amounts that can inhibit the proliferation of MCF-7 cells, as illustrated in Table 4.

The growth curve in Figure 2 shows a variation in the growth curve between the solvents used. However, the AS growth chart with their ethyl acetate solvent decreased growth at a concentration close to their growth point of concentration of 62 µg/ml. Table 1 provides evidence of a 50% growth barrier concentration for their MCF-7 cell population. The IC50 calculation yielded a score of 62 µg/ml, indicating a patient inhibition of the growth of MCF-7 breast cancer cells.

The AS continued fractional testing with three repetitions. Inter-fraction variation results indicated a reduction in cell proliferation of their MCF-7 line corresponding to an increase in the concentration of the sample utilized (31.25, 62.5, 125, 250, 500, 1,000 µg/ml) (Figure 3). The N-hexane and ethyl acetate fractions exhibited potential anticancer IC50 values of 49 µg/ml (strong) and 89 µg/ml (strong), respectively (Table 2); the comparison of growth curves among AS fractions indicates a decline in growth at varying concentrations. Figure 4 shows the growth curve with variations between the fractions obtained. However, the growth graph of AS with N Hexane fraction decreased growth at a concentration close to the growth point concentration of 49 µg/ml. Table 4 provides evidence of a growth barrier concentration of 50% for the MCF-7 cell

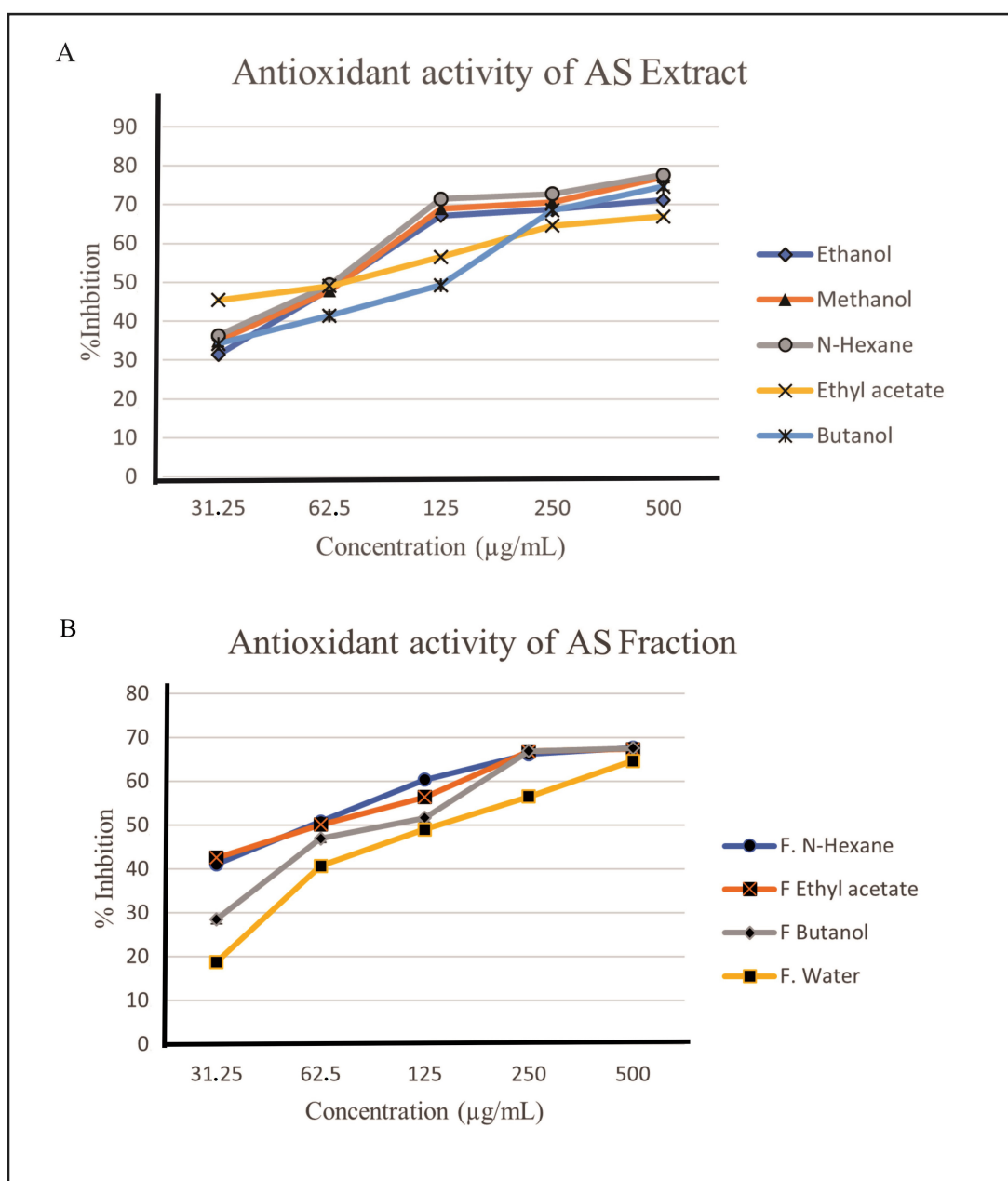


Figure 1. Concentration-dependent DPPH inhibition activity of AS **A.** Extract of AS, **B.** Fraction of AS.

Table 1. The levels of antioxidant activity, TFC, and TPC against AS extract and fractions.

	Sample	Antioxidant activity IC50 (µg/ml)	TPC (mg GAE/g)	TFC (mg QE/g)
Extraction AS	Ethanol	42.7	21.59	15.54
	Methanol	46.3	18.90	15.95
	N-Hexane	35.53	25.61	29.50
	Ethyl acetate	40.3	22.73	17.27
	Butanol	302.4	14.89	14.96
Fraction AS	Butanol	320.4	10.89	15.51
	Ethyl acetate	56.9	21.69	26.93
	N-Hexane	42.4	25.31	31.00
	Water	435.2	18.22	15.68

Table 2. Pearson correlation between antioxidant activity, with fenolic content and flavonoid content.

	Extract of AS			Fraction of AS		
	Antioxidant activity	TFC	TPC	Antioxidant activity	TFC	TPC
Antioxidant Activity	1			1		
TPC	-0.9*	1		0.407	1	
TFC	0.9*		1	0.898		1

TFC = Total flavoinoiid content; TPC: Total pheinoilic cointeint; *significant at *p*-value <0.05.

Table 3. IC₅₀ value of AS extract with several different solvents.

Tested compound (AS)	IC ₅₀ (µg/ml)	Interpretations
N-Hexane extract	101	Weak
Ethyl acetate extract	62	Strong
Ethanol extract	416	No potentially
Methanol extract	301	No potentially

Table 4. IC₅₀ value of AS fraction with several different fractions.

Tested compound (AS)	IC ₅₀ (µg/ml)	Interpretations
N-Hexane fraction	49	Very strong
Ethyl acetate fraction	89	Strong
Butanol fraction	8817	No potentially
Water fraction	254	No potentially

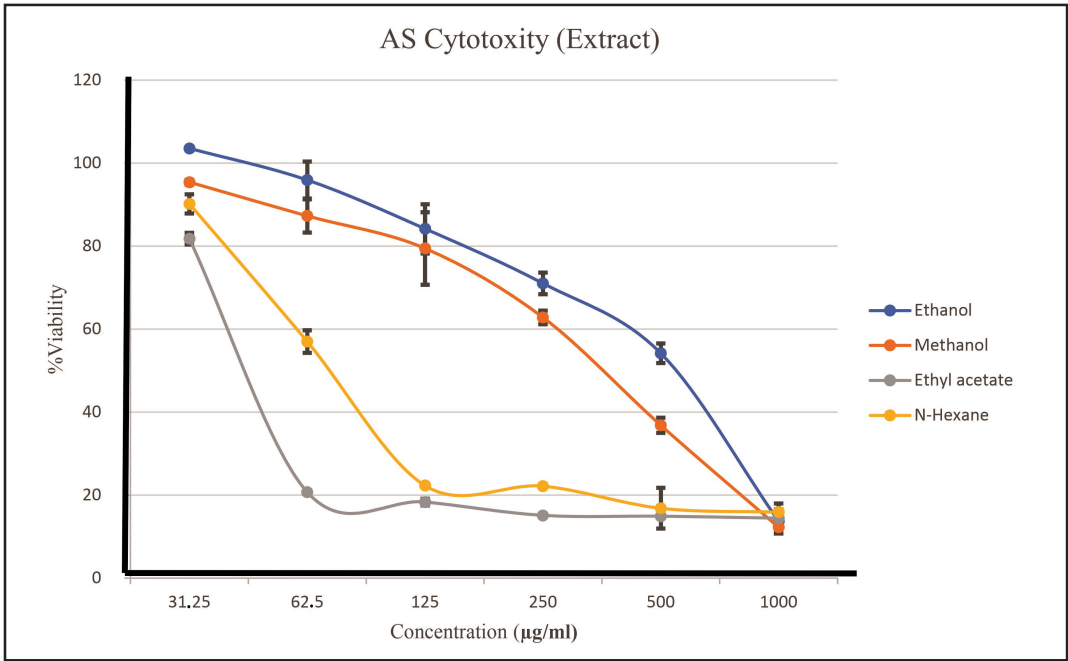


Figure 2. Comparison of growth curves of all extracts of AS.

population. The IC₅₀ calculation resulted in a score of 49 µg/ml, which indicates the inhibition of breast cancer cell growth against the MCF-7 cell line.

Table 4 shows the calculation of the IC₅₀ value for each fraction, which is used to determine precisely what concentrations can inhibit the growth of MCF-7 cells.

A reduction in cell viability is noted at doses beginning at 5 µg/ml, with data indicating a diminishing growth curve if MCF-7 cells as their extract concentration escalates. The n-hexane and ethyl acetate extracts inhibit 50% MCF-7 cell proliferation at 62.5–1000 µg/ml. This indicates that these chemicals possess cytotoxic properties. The reduction in viability signifies that the active chemicals in their extract can compromise cell integrity and hinder the multiplication of cancer cells. These findings correspond with prior studies indicating that plant extracts can be anticancer agents,

influencing apoptotic pathways and obstructing their cancer cell cycle [41]. Consequently, their subsequent stage involves further screening to isolate the bioactive compounds accountable for this anticancer activity and executing docking analysis to investigate the connections between these compounds and molecular targets. This discovery establishes a robust basis for advancing plant-derived cancer therapeutics, which may offer an alternative to traditional treatments that frequently include detrimental side effects.

Data variation among fractions, conducted with three repetitions, indicates a notable reduction in the proliferation of MCF-7 cells as the concentration of their sample escalates, particularly from 31.25 to 1,000 µg/ml. The reduction in cell viability signifies that their active chemicals in the n-hexane and ethyl acetate fractions effectively impede cancer cell proliferation. The IC₅₀ value for their n-hexane fraction is 49

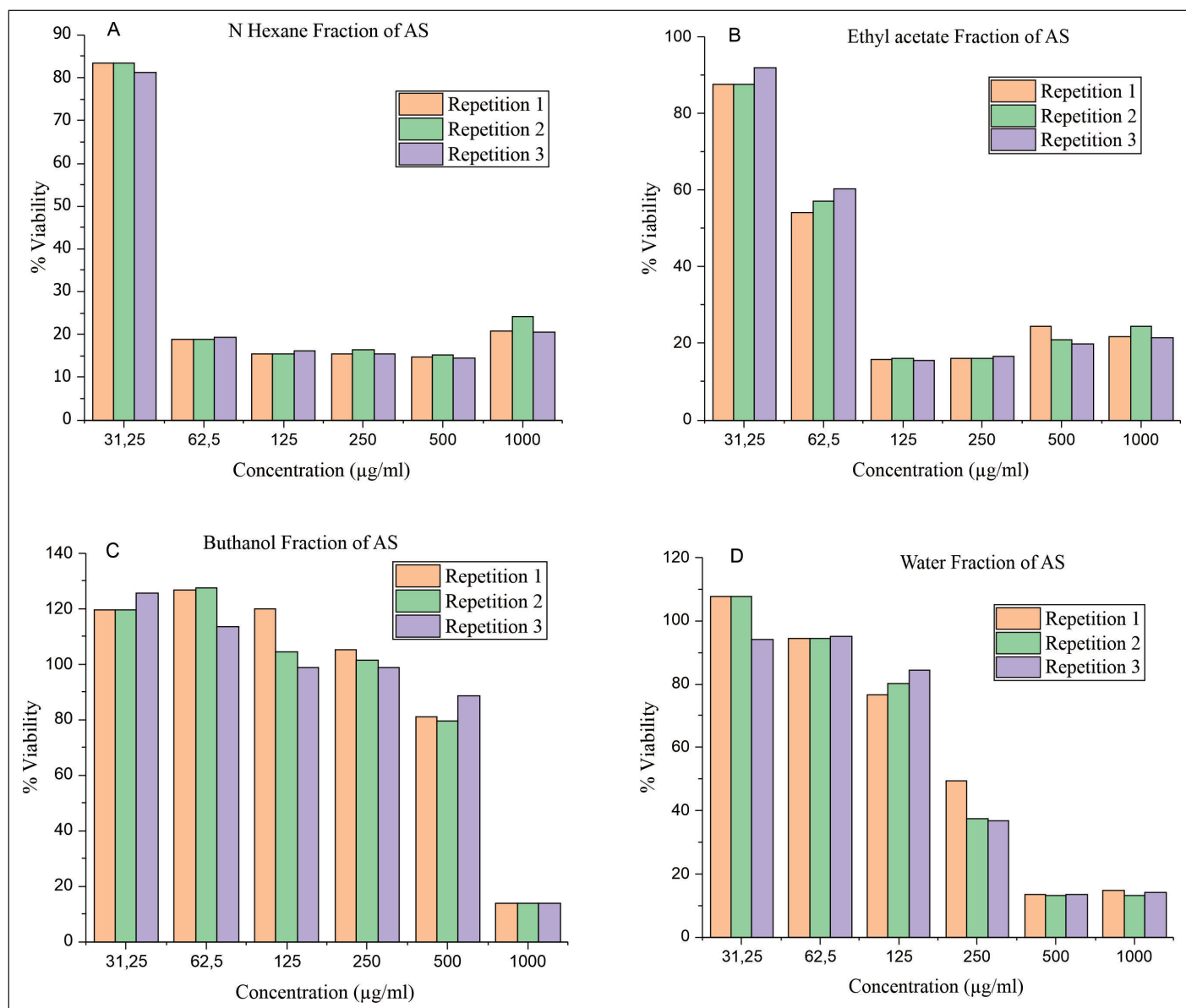


Figure 3. The MCF-7 cell growth curve is compared to several AS fractions: (A) N-Hexane fraction of AS, (B) Ethyl acetate fraction of AS, (C) Butanol fraction of AS, and (D) Water fraction of AS.

µg/ml; their ethyl acetate fraction is 89 µg/ml, demonstrating significant anticancer activity in both fractions. These findings align with prior studies indicating that extracts from specific plants exhibit cytotoxic effects against various cancer cell types, including MCF-7, via mechanisms such as apoptosis induction and cell cycle inhibition [42,43]. This discovery underscores the necessity of further investigating the bioactive chemicals in that fraction, which may be developed as novel therapeutic agents for cancer treatment.

Hexane extract and fractions tend to extract lipophilic compounds such as specific terpenoids and flavonoids known to have anticancer activity. These compounds can interact with the cancer cell membrane, disrupt the cell proliferation process, and trigger apoptosis (programmed cell death). On the other hand, although polar and capable of extracting polar

compounds, ethanol, and methanol extracts may not contain high concentrations of bioactive compounds that effectively inhibit the growth of MCF-7 cells. Previous research by Zhang *et al.* [44] showed that lipophilic compounds have a better affinity for targeting signaling pathways involved in cancer growth [45]. This may explain why ethanol and methanol-based extracts do not exhibit the same potential. Butanol solvent on extract and fraction with medium polarity may also not contain the optimal active compounds to inhibit cancer cell growth. According to Chehelgerdi [46] bioactive compounds isolated from medicinal plants often have varying efficiencies depending on the type of solvent used. In this case, the components extracted with butanol may not be sufficiently effective in targeting the active oncogenic pathways in MCF-7 cells [47].

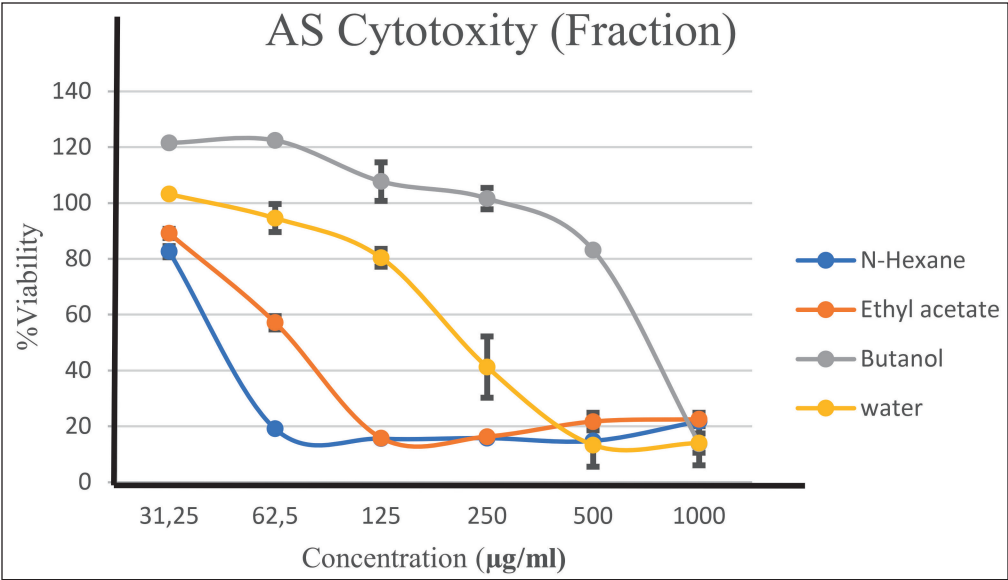


Figure 4. Comparison of growth curves of all fractions of AS.

Table 5. Prediction of phytochemical compounds Fraction of ethyl acetate AS.

No	RT (min)	Product compound	Measured (m/z)	Exact mass	m/z cloud best match confidence (%)	Product ions (m/z)
1	14.4	α -Linolenic acid	279.23	278.23	100	279.23, 261.21, 243.21
2	13.4	Galaxolidone	272.18	271.18	93.1	176.11, 255.12, 255.17
3	10.3	Pinocembrin	257.08	256.08	95.9	257.08, 153.01, 131.04
4	6.8	Afzelin	431.09	432.09	95.9	285.04, 284.03, 255.02

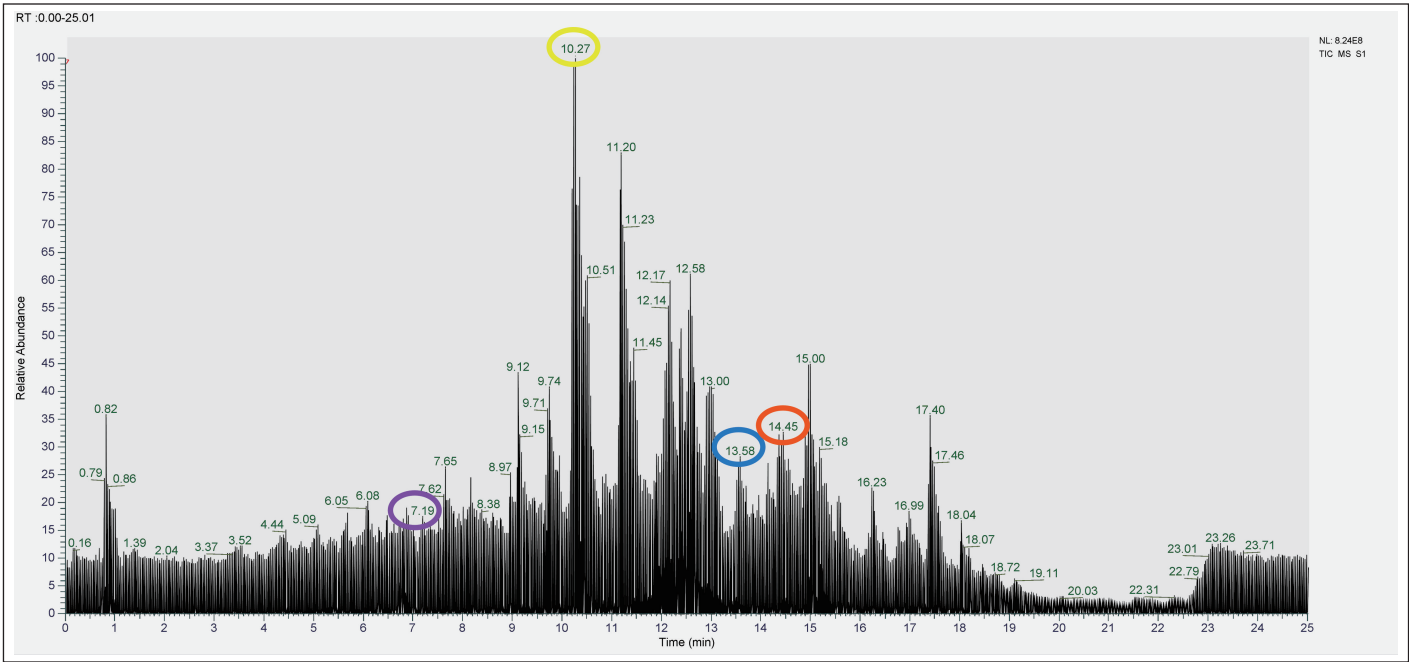
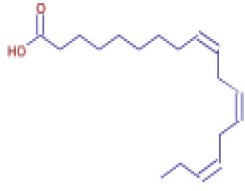
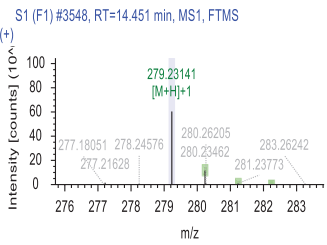
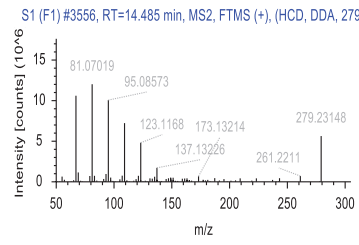
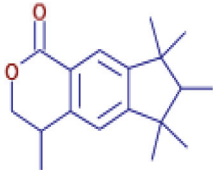
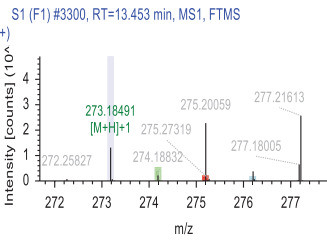
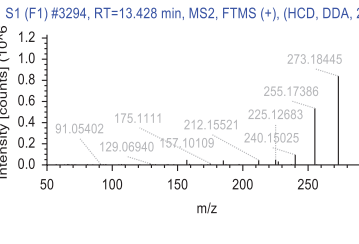
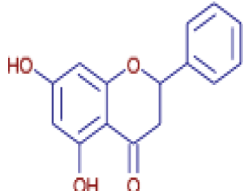
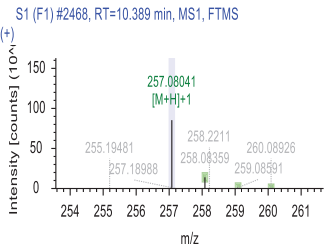
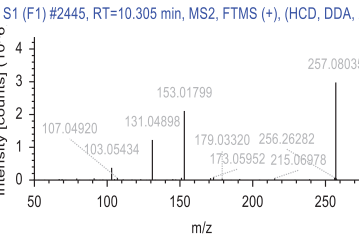
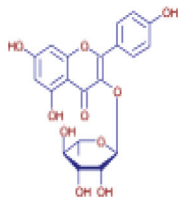
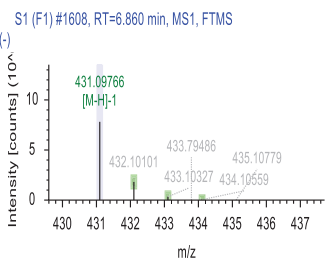
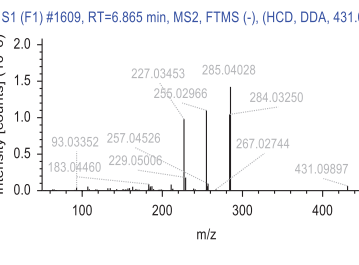


Figure 5. TIC graph in phytochemical screening results (Red circle: α -Linolenic acid; Blue Circle: Galaxolidone; Yellow Circle: Pinocembrin; Purple Circle: Afzelin).

Table 6. Chemical formula and MS-MS spectra.

No	Structure	MS spectra-1	MS spectra 2
α-Linolenic acid			
1			
Galaxolidone			
2			
Pinocembrin			
3			
Afzelin			
4			

Analysis of AS N-Hexane fraction compounds with LC-HRMS

This study uses an LC-HRMS analysis to identify the sensitivities present in the AS N-Hexane fraction. LC-HRMS enables comprehensive molecular fingerprinting in a sample, following up on the procedure of Windarsih *et al.* [38]. The analysis's results indicate the predicted relationship between the identified subject and the m/z cloud base data. A high-resolution spectrometer analyses various molecules using the MS and MS/MS spectra presented in the mzCloud basis data. This spectrum can be used as a reference when looking up uncommon words. Identification of molecules is based on high-resolution data from single molecules and all fragments of molecules. The

Compound Discover tool compares the fragmentation patterns and molecular ions with those found in the m/zCloud database to determine the best match. In the case of a compound match, the software will show ranges from 0 to 100, with a higher score indicating a more significant number of the best matches of the number match of ions [38].

A total of 4 compounds were successfully filtered by considering their best value to be from cloud best match confidence, with a minimum value ranging from 95% to 100%, to proceed with silicon analysis and obtain screening results in the form of potential compounds that can reduce cancer cell proliferation. Screening Results are shown in Table 5.

Information regarding the retention time (RT) on the total ion current (TIC) graph in phytochemical screening results using LC-HRMS is essential for identifying and analyzing compounds. Each compound has a characteristic RT, so the peaks that appear on the TIC graph can be used to determine when a compound elutes from the chromatography column, as shown in Figure 5.

The LC HRMS testing results indicated that four substances were accurately identified and selected based on optimal mzCloud match values, with scores between 95% and 100%. This filtering procedure is essential for their methods, such as silicon analysis, to identify possible chemicals limiting cancer cell proliferation. These characteristics derived from existing, including Measured (m/z), Exact mass m/z, Cloud Best Match confidence (%), and Product coins (m/z), are essential for their identification of pertinent substances. The measured m/z offers insights into the mass-to-charge ratio, whereas the exact mass m/z facilitates precise compound identification [48]. The Cloud Best Match confidence (%) signifies the degree of assurance concerning the data match, guaranteeing that the chosen compounds are representative and pertinent. Production (m/z) data elucidate the fragmentation of compounds, adding to the comprehension of their chemical structure and potential biological action. By synthesizing the outcomes from LC HRMS and in silico analysis, researchers may effectively forecast interactions between drugs and molecular targets, thereby expediting the development of anticancer medicines. Our research findings align with prior studies demonstrating that a multidisciplinary approach in drug development, incorporating LC HRMS technology and in silico analysis, can improve the efficacy and safety of cancer therapies derived from natural compounds [49,50].

The LC-HRMS test found a group of phytochemicals that are very interesting. These are Galaxolidone, α -linolenic acid, pinocembrin, and afzelin. The m/z values indicate their easy identification. Galaxolidone (279.23 m/z) and α -linolenic acid (272.18 m/z) were detected with perfect Cloud Best Match. Confidence values, 100% and 93.1%, respectively, indicate that these compounds have strong potential to provide biological effects. Also, pinocembrin (257.08 m/z) and afzelin (431.09 m/z) have high confidence values (95.9%), which means that these compounds may help the extract work as expected in the pharmaceutical field.

Galaxolidone is a phytochemical compound synonymous with estradiol and is an aromatic compound that can reduce inflammation and protect cells from damage. It can stop cancer cells from multiplying by controlling signaling pathways in the cell cycle and induced apoptosis mechanism [51]. *Galaxolidone* or Galaxolide is a sesquiterpene lactone, a chemical frequently occurring in flora. It is recognized for its possible anti-inflammatory and antibacterial characteristics [52]. Galaxolidone has been extracted from multiple plant species, including those within their Asteraceae family. Flavonoids are recognized for their antioxidant characteristics and possible health advantages [53]. Pinocembrin has been investigated for its prospective anti-inflammatory, neuroprotective, and anticancer properties [54]. α -Linolenic acid, an omega-3 fatty acid, is also known to help fight cancer. Studies have shown

that this compound can cause apoptosis in MCF-7 cells and stop cancer cells from spreading and migrating. Various plants contain pinocembrin, a flavonoid that exhibits cytotoxic effects against MCF-7 cells and can inhibit signaling pathways in cancer cell growth [55].

Afzelin, a flavonoid glycoside, possesses significant potential in inhibiting the growth of MCF-7 cells. Previous research has shown that afzelin can induce cell death through apoptosis and disrupt the proliferation process of cancer cells [56,57]. Overall, these compounds show potential as promising anticancer agents, and further research is needed to understand their mechanisms of action and effectiveness in cancer therapy. Because there is much evidence that these compounds can fight cancer, AS can be seen as a valuable natural source for making cancer treatments that work better and are safer.

CONCLUSION

With a value of 35.53 $\mu\text{g/ml}$, total phenol is 25.61 mg GAE/g, and total flavonoid is 29.50 mg QE/g, this study shows that N-hexane extract may be a better antioxidant than other test materials. This is true for both the extract and fraction of AS. These data are also supported by the inhibition of MCF-7 cell growth with an IC₅₀ value of 42 $\mu\text{g/ml}$ in the n-hexane extract, which is the best IC₅₀ value. This is a further indicator for evaluating the LC-HRMS phytochemical profile of N-Hexane AS extract, and four phytochemical compounds are obtained. Galaxolidone, linolenic acid, pinocembrin, and afzelin.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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