Demonstrating the antioxidant, antibacterial, and anticancer properties of the Achillea fragrantissima shrub growing in Riyadh, Saudi Arabia

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Abstract

Achillea fragrantissima is a known medicinal plant in traditional Saudi culture. The current study aimed to evaluate the chemical composition of *A. fragrantissima* and its biological activity as a possible antioxidant, antibacterial, and anticancer agent. The phenolic content of *A. fragrantissima* was studied using Fourier transform infrared spectroscopy (FTIR) and gas chromatography/mass spectrometry (GC/MS). The antibacterial activity was investigated using the well-diffusion method. The anticancer was investigated against A594 lung cancer cells by MTT, annexin V/Propidium iodide assay, Cell cycle analysis, and Western blotting. The chemical analysis showed that *A. fragrantissima* includes volatile oils, fatty acids, and other aromatic chemicals. *A. fragrantissima* ethanolic extract (AFEE) had a 38% scavenging of free radicals at 12 µg/ml and 48% at 100 µg/ml ($p < 0.001$). Using a 40% dose, the antibacterial test revealed substantial efficacy against all species, particularly *S. aureus*. The cytotoxic effect showed dose-dependent growth inhibition of A594 cells with an IC_{50} of 14.01 µg/ml. At 20 µg/ml, AFEE caused 33.27% of cellular apoptosis. At 40 µg/ml, G2/M phase arrest increased to 36.77% ($p < 0.001$). Mechanistically, AFEE reduced the anti-apoptotic protein Bcl-2 by 56.14% and increased caspase-3 by 66.27% while boosting Cyclin B levels by 74.35%. These findings emphasize AFEE's potential as an antioxidant, antibacterial, and anticancer agent.

Introduction

Saudi Arabia's flora is diverse, with herbs accounting for 70%, shrubs for 27%, and trees for only 3% [1]. Compounds obtained from numerous herbal treatments exhibited radical scavenging and antibacterial properties. They can be considered the human body's defense against pathogens and cellular oxidation. Because of their antibacterial, antiviral, anticancer, and antioxidant properties, these components are useful raw materials for producing many types of herbal medicine [2, 3]. As a result of the extracted herbal active components, various studies have focused on developing antibacterial, antiviral, and anticancer medications with fewer side effects [4, 5].

Achillea is a genus of more than 130 flowering and perennial plants native to Europe and temperate Asia. These plants are recognized by their hairy, scented leaves and flat clusters of little flowers at the stem's tip. Because these blooms appear in various colors, several varieties make appealing garden plants [6]. *Achillea fragrantissima* is a medicinal and aromatic shrub widely planted in Saudi Arabia. It has been stated to have medical applications and is utilized in traditional cultures and folkloric medicine among Saudis [7]. *A. fragrantissima* has long been used to treat fever and chronic illnesses such as arthritis and diabetes, with anti-inflammatory, antioxidant, and antiproliferative effects on cancer cells [8].

A. fragrantissima is a small, strongly scented, white-woolly shrub that grows to a height of 30–60 cm. This yarrow is native to North Africa and the Middle East (including Egypt, Jordan, Palestine, Syria, Lebanon, Iraq, and Saudi Arabia). It flourishes in deserts and semi-deserts [9]. Its ability to grow in arid environments underlines its potential application in sustainable agriculture, ecological restoration, and even extraterrestrial settlement, where resource-efficient medicinal plant production is critical.

Currently, phrenological strategies using *A. fragrantissima* pure extracts or active metabolites to overcome the resistance of more dangerous microbes are widespread. The use of *A. fragrantissima* extracts in ethanol reduces metal ions to the base metal in a relatively short amount of time, at room temperature and pressure, and on a large scale [10]. *A. fragrantissima* has received attention for its powerful anticancer and antioxidant effects. This plant, which is high in bioactive substances including flavonoids, phenolics, and terpenoids, has strong free radical-scavenging activity, which helps protect cells from oxidative damage, a major cause in cancer development. Extracts of *A. fragrantissima* have been proven in studies to induce apoptosis, decrease cancer cell growth, and restrict migration in a variety of cancer models, indicating their potential as a natural therapy [11, 12]. Its antioxidant qualities enhance its anticancer benefits by lowering oxidative stress and regulating critical signaling pathways involved in tumor growth.

A. fragrantissima's anti-inflammatory and neuroprotective qualities make it a promising candidate for rehabilitation and disability research, in addition to cancer research. Many chronic illnesses that cause disability, including arthritis, neurological disorders, and muscle deficits, have been related to oxidative stress and inflammation [13]. *A. fragrantissima*'s traditional use in pain reduction and inflammatory control suggests that it has the potential to be used in the development of novel treatments in rehabilitation. For example, bioactive chemicals from this plant may aid in tissue regeneration, reduce muscle and joint inflammation, and promote neurological recovery after injuries or neurodegenerative disorders such as Parkinson's and Alzheimer's [14].

A. fragrantissima's pharmacological capabilities and resilience in severe desert climates make it not only a prospective option for cancer prevention and therapy, but also a possible important species for future bioregenerative life support systems. Research into its bioactive chemicals and medical applications is consistent with the larger goals of inhabitation research, which emphasizes the importance of sustainable, self-sufficient ecosystems in maintaining human health in remote or severe locations. So, the study seeks to provide scientific justification for the traditional use of *A. fragrantissima*, growing in Saudi Arabia, by examining its chemical composition and biological activity, as well as to investigate its potential as an antioxidant, antibacterial, and anticancer agent represents its rationale.

Results

Functional groups analysis of AFEE

The phytochemical properties of *A. fragrantissima* ethanolic extract (AFEE) were analyzed using FTIR and GC/MS techniques. FTIR spectroscopy identified the presence of seven distinct functional Groups. A strong, broad peak at 3276 cm^{-1} was identified as the O-H stretching group, which indicated the presence of a carboxyl alcohol. Another broad peak was detected at 2985 cm^{-1}, which was translated as the N-H Stretching group, indicating an Amine salt, while a strong peak at 1733 cm^{-1} was more likely a C = O Stretching Aldehyde. Finally, two medium peaks at 1591 cm^{-1} and 1594 cm^{-1} were likely to be N-H

Bending Amine, while the two medium peaks at 1249 cm^{-1} and 1026 cm^{-1} were identified as C-N Stretching Amine groups (Fig. 1).

Phenolic compounds analysis of AFEE

GC/MS analysis revealed a variety of bioactive compounds in AFEE, including the fatty acid esters of cis-3-Hexenyl caproate (23.82%) and Ethyl (E)-3,4,4-trimethylpent-2-enoate (11.65%). Other major constituents included the di-alkyl ether E-1-Methoxy-4-hexene (11.67%), the long-chain fatty acids of Palmitic acid (10.06%), cis-vaccenic acid (8.67%), and Stearic acid (6%). Minor terpenoid constituents were reported and included R-Citronellene (0.4%), Norbornane (0.28%), Caryophyllene oxide (0.28%), and Norpatchoulenol (0.62%) (Table 1).

Table 1
Phenolic constituents of AFEE.

Phenolic compound	Formula	Molecular weight	Area (Ab*s)	Peak area (%)	Classification
2,5-dimethyl-2,3,4-Hexatriene	C_8H_{12}	108.094	117263	0.88	Branched unsaturated hydrocarbons
(1S,2S)-Cyclohexane-1,2-diyldimethanol	$C_8H_{16}O_2$	144.115	33936	0.26	Primary alcohols
5,9-dimethyl-4,8-Decadien-3-ol	$C_{12}H_{22}O$	182.167	16781	0.13	Monoterpenoids
Cyclohexylacetone	$C_9H_{16}O$	140.12	40075	0.3	Ketones
2-Dodecanone	$C_{12}H_{24}O$	184.183	29956	0.23	Ketones
2-Pentyn-1-ol	C_5H_8O	84.058	30495	0.23	Primary alcohols
2-Methylthiazoline	C_4H_7NS	101.03	45219	0.34	Thiazoles
Caproic acid	$C_6H_{12}O_2$	116.084	35006	0.26	Fatty acids
m-Cymene	$C_{10}H_{14}$	134.11	53866	0.41	Cumenes
+-α-Thujone	$C_{10}H_{16}O$	152.23	55361	0.42	Monoterpenoids
(1S)-1-methylcyclohex-2-en-1-ol	$C_7H_{12}O$	112.089	1106850	**8.34**	Tertiary alcohols
3-Methyltetrahydrofuran	$C_6H_{10}O$	98.073	34770	0.26	Tetrahydrofurans
N-Formylpiperidine	$C_6H_{12}N_2S$	113.084	60428	0.46	Piperidines
3,5-Dihydroxy-6-methyl-2H-pyran-4(3H)-one	$C_6H_8O_4$	144.042	242731	**1.83**	Dihydropyranones
R-Citronellene	$C_{10}H_{18}$	138.141	53685	0.4	Monoterpenoids
cis-3-Hexenyl caproate	$C_{12}H_{22}O_2$	198.162	3163564	**23.82**	Fatty acid esters
E-1-Methoxy-4-hexene	$C_7H_{14}O$	114.104	1549339	**11.67**	Dialkyl ethers
(E)-3-Undecen-5-yne	$C_{11}H_{18}$	150.141	145947	1.1	Enynes
Ethyl (E)-3,4,4-trimethylpent-2-enoate	$C_{10}H_{18}O_2$	170.131	1547363	**11.65**	Fatty acid esters
1-(1-methylene-2-propenyl)-cyclopentanol	$C_9H_{14}O$	138.104	33680	0.25	Cyclopentanols

Phenolic compound	Formula	Molecular weight	Area (Ab*s)	Peak area (%)	Classification
4,4-Dimethyl-2-pentyne	C_7H_{12}	96.094	343739	2.59	Alkynes
Furfuryl amine	C_5H_7NO	97.053	34095	0.26	Aralkylamines
3,5-Octadien-2-one	$C_8H_{12}O$	124.089	457042	**3.44**	Unsaturated ketones
3-hydroxy-2(2-oxopropyl)-2-cyclohexene-1-one	$C_9H_{12}O_2$	152.084	105798	0.8	Ketones
Norbornane	$C_{10}H_{18}$	138.141	37368	0.28	Monoterpenoids
Ascaridole	$C_{10}H_{16}O_2$	168.115	54515	0.41	1,2-dioxanes
Caryophyllene oxide	$C_{15}H_{24}O$	220.183	37679	0.28	Sesquiterpenoids
8-Methoxy- [1, 2, 4] triazolo[4,3-a] pyridine-3-thiol	$C_6H_5N_3S$	181.031	101449	0.76	Triazolopyridines
D-glycero-D-gulo-Heptonic acid, delta-lactone	$C_7H_{12}O_7$	208.058	50611	0.38	Monosaccharides
Palmitic acid	$C_{16}H_{32}O_2$	256.24	1336396	**10.06**	Fatty acids
cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.256	1150818	**8.67**	Fatty acids
Stearic acid	$C_{18}H_{36}O_2$	284.272	796602	**6**	Fatty acids
15-hydroxy-pentadecanoic acid	$C_{15}H_{30}O_3$	258.219	45385	0.34	Fatty acids
Norpatchoulenol	$C_{14}H_{22}$	190.172	82694	0.62	Tricyclic terpenoid
2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.277	247888	1.87	Monoacylglycerols

The antibacterial activities of AFEE

The antimicrobial activity of AFEE was evaluated using three different doses against various bacterial species, including *S. aureus, P. aeruginosa, B. subtilis*, and *E. coli.* The inhibition zone diameters were compared to erythromycin as a positive control. AFEE significantly inhibited the growth of the gram-positive species *S. aureus* and *B. subtilis* ($p < 0.001$). For *E. coli*, only the highest dose of AFEE (40 µg/ml) was effective ($p < 0.001$). Conversely, *P. aeruginosa* displayed the greatest resistance (Table 2).

Table 2
Antibacterial effects of AFEE (diameter of zone of inhibition (mm)).

Species		DMSO	Cephalexin	10 µg/ml	20 µg/ml	40 µg/ml	MIC
E. coli	Mean ± SD	0	18 ± 0	0	0	11.7 ± 0.6	> 40 µg/ml
	Median (Min-Max)	0	18 (18–18)	0	0	12 (11–12)	
	% Inhibition	0%	20.45%	0%	0%	13.26%	
	P-value	1	< 0.001*	1	1	< 0.001*	
B. subtilis	Mean ± SD	0	25.8 ± 0.8	0	9.8 ± 0.6	13.2 ± 0.3	15 µg/ml
	Median (Min-Max)	0	26 (25-26.5)	0	10 (9–10)	13 (13-13.5)	
	% Inhibition	0%	29.36%	0%	10.98%	14.96%	
	P-value	1	< 0.001*	1	1	< 0.001*	
P. aeruginosa	Mean ± SD	0	19.7 ± 0.6	0	0	0	> 40 µg/ml
	Median (Min-Max)	0	20 (19–20)	0	0	0	
	% Inhibition	0%	22.35%	0%	0%	0%	
	P-value	1	< 0.001*	1	1	1	
S. aureus	Mean ± SD	0	26.8 ± 0.8	0	13.2 ± 0.3	14.8 ± 0.8	15 µg/ml
	Median (Min-Max)	0	27 (26-27.5)	0	13 (13-13.5)	15 (14-15.5)	
	% Inhibition	0%	30.49%	0%	14.96%	16.86%	
	P- value	1	< 0.001*	1	1	< 0.001*	

*Significant at $p < 0.05$. MIC: Minimum Inhibition Concentration.

AFEE is an antioxidant agent

The Scavenging activity assay results revealed that showed significant free-radical scavenging of almost 38% by 12 µg/ml of AFEE and reached more than 48% at 100 µg/ml, in comparison to that of ascorbic

acid ($p < 0.001$) (Fig. 2).

AFEE increased the cytotoxicity of A594 cells

The MTT assay showed potential cytotoxicity activity that affected the growth and proliferation of A594 cells. The lowest significant inhibitory concentration was 12.5 µg/ml, which induced about 35% inhibition, whereas about 73% inhibition was obtained by 200 µg/ml ($p < 0.001$). The calculated IC_{50} was 14.0.1 µg/ml (Fig. 3).

AFEE-triggered apoptosis of A594 cells

As shown in Fig. 4, AFEE increased the cellular apoptosis of A594 cells at 20 µg/ml to 33.27% compared to the control (1.44%). Most cells (22.85%) underwent early apoptosis at a 20 µg/ml dose, while only 10.42% were processed to late apoptosis or complete cell death. At the highest concentration of 40 µg/ml, it was noticed that the total apoptotic effect seemed to decrease; however, as noticed in Fig. 4A, the number of dots was lower than those in the control, 10 µg/ml, and 20 µg/ml. This is because most cells shrieked and shifted to the debris area near the zero point, located outside the gated area of the FSC and SSC dot blot.

AFEE induced G2/M phase arrest and increased cellular death

In comparison to the untreated control cells, it was noticed that the G2/M phase levels increased significantly from 19.18–27.97% ($p < 0.05$), 31.17% ($p < 0.001$), and 36.77% ($p < 0.001$) in the cells treated with 10, 20, and 40 µg/ml of AFEE, respectively (Fig. 5). On the other hand, a dramatic decrease in the G0/G1 phase levels was observed. Furthermore, the number of cells arrested at the Sub G stage increased significantly ($p < 0.001$), which indicated increased cellular death.

Effect of AFEE on the cellular proteins integrated in the apoptotic and cell cycle pathways

To confirm the apoptotic effect of AFEE in A594 cells, the expression of the apoptotic suppressor (Bcl-2) and apoptotic activator (caspase 3) was in a dose-dependent manner (Fig. 6). Bcl-2 levels decreased by 56.14%, while the expression levels of Caspase 3 increased by 66.27% at the dose of 40 µg/ml, compared to the control ($p < 0.001$).

The levels of Cyclin B increased up to 74.35% at the highest dose of AFEE, compared to the control, which might explain how AFEE impairs Cyclin B, causes its accumulation, and induces a cellular arrest at the G2/M phase ($p < 0.001$).

Discussion

The current study investigated the phytochemical properties of *A. fragrantissima* by FTIR and GC/MS analysis to evaluate the common functional groups and phenolic constituents, respectively. The FTIR spectrum confirmed that AFEE was rich in five groups of amine salts (N-H bending, N-H stretching, and

C-N stretching), a strong peak of aldehyde (C = O stretching), and a strong peak of carboxylic/alcoholic group (O-stretching) at 3276 cm^{-1}, but no alkenes. Several studies on different plants found comparable chemical makeup of an *Asteraceae* family member. FTIR examination confirmed the presence of alkyl halides, alkanes, alkenes, aldehydes, and amide groups in recent research on the aqueous extract of the leaves of *Matricaria chamonbmilla* [15]. Another study confirmed the existence of aromatic compounds containing hydroxyl and carbonyl groups by FTIR examination of *Centaurea cyanus* flower extract with additional acetyl, C = C, and C = O functional groups [16]. In another investigation, FTIR spectroscopy was used to determine the existence of the C-H stretch, C = O stretch, O-H stretch, and C-O stretch functional groups in the essential oils of *Arnica montana, Echinacea purpurea, Calendula officinalis, Tagetes erecta*, and *Chamomilla recutita* [17].

The chemical composition of AFEE was phytochemically examined by GC/MS analysis. The GC/MS analysis revealed the presence of multiple phenolic biomolecules, including phenolics, fatty acids, ketones, terpenoids, and antioxidants. The GC/MS results showed that the most represented compounds in the *A. fragrantissima* extract were cis-3-Hexenyl caproate (23.82%), E-1-Methoxy-4-hexene (11.67%), Ethyl (E)-3,4,4-trimethylpent-2-enoate (11.65%), Palmitic acid (10.06%), cis-Vaccenic acid (8.67%), 1-methylcyclohex-2-en-1-ol (8.34%), and Octadecanoic acid (6%). Similar findings were reported by Qader *et al.* (2018), in which the phytochemical constituents of the essential oils of *A. fragrantissima* leaves were reported as m-cymene (0.8%) and α-Thujone (1.15%) [18]. In the study conducted by Alsohaili and Al-Fawwaz (2014), the GC/MS analysis of the *A. frangantissima* essential oil revealed that the major constituents were Artemisia ketone (20%), α-Thujone (12%), Carvacrol (13%), p-Cymene (2%), and β-Sesquiphellandrene (15%), which is quite similar to the current findings [19]. Another study showed that the major fractions of the essential oil of *A. fragrantissima* were (1S, 4S, 5R)-1-isopropyl-4-methylbicyclo [3.1.0] hexan-3-one (cis-thujone) and 3, 3, 6-trimethyl-1, 5-heptadien-4-one (artemisia ketone), whereas for *Achillea biebersteinii*, cis-ascaridole and P-cymene were the most represented **[20].** Furthermore, for the oil of *Achillea santolina*, fragranyl acetate and 1,6-dimethyl-1,5-cyclooctadiene represented the highest concentrations, while chamazulene and b-pinene were more common in *Achillea millefolium* **[20].** A previous study analyzing the constituents of the essential oil of different members of the *Asteraceae* family reported that ascaridole (43.22%), iso-ascaridole (37.17%), and 1,8-cineole (45.2%) were detected in *A. biebersteinii* [21], chamazulene (52.60%) in *Achillea kellalensis* [22], chrysanthenone (38.8%), trans-carveol (27.5%), neoiso-dihydrocarveol acetate (25.2%), filifolone (19.7%), apinene (11.8%), trans-piperitol (11.7%), (E)-caryophyllene (11.2%), (E)-nerolidol (10.8%), and lavandulyl acetate (26.19%) in *Achillea wilhelmsii* **[23].** All these reports and studies confirm the current findings about the robust phytochemical composition of different extracts of *A. fragrantissima.*

AFEE showed substantial antioxidant activity, with 38% activity at 12 µg/ml and over 48% at 100 µg/ml, comparable to ascorbic acid. These findings are consistent with earlier research on other *Achillea* species, emphasizing their potent antioxidant effects due to high phenolic and flavonoid content. For example, a study revealed that *Achillea atrata* L. and *Achillea millefolium* L. are rich in luteolin, apigenin, centaureidin, and nevadensin, which are known as their effective 2,2-diphenyl-picryl hydrazyl radical scavengers [24]. This shows that different species in the *Achillea* genus have a similar bioactive profile,

which contributes to their strong antioxidant effects. Comparable research from Saudi Arabia highlights the high antioxidant activity of natural medicinal herbs. Research of essential oils extracted from *A. fragrantissima* grown in Saudi Arabia and Egypt discovered substantial radical-scavenging activity, with IC_{50} values of 30.94 and 28.72 mg/L, respectively [25]. This highlights the importance of *A. fragrantissima* as a prospective natural source of antioxidants. In the study conducted by Elsharkawy *et al.* (2020), the antioxidant activity of *A. fragmentisma* growing in different regions of Saudi Arabia significantly differed, where those from the Arar and Tabuk regions exhibited their scavenging activities at IC_{50}s of 0.21 ± 0.01 and 1.12 ± 0.03 mg/ml, respectively [8]. These commonalities show that plants in this family share bioactive chemicals that provide variable antioxidant effects, which might be affected by weather parameters, and that supports their usage in traditional medicine. The observed antioxidant potential of AFEE can be attributable to its high phenolic and flavonoid content, as corroborated by prior phytochemical research on *A. fragrantissima*. The inclusion of volatile oils, fatty acids, and aromatic components boosts its free radical scavenging activity. Given the growing interest in plant-based antioxidants for pharmaceutical and nutraceutical uses, *A. fragrantissima's* high activity supports its promise as a natural alternative to synthetic antioxidants.

In the current study, three doses of AFEE were used to assess their antibacterial activities on the growth of different species (*S. aureus, P. aeruginosa, B. subtilis*, and *E. coli*). The inhibition zone diameter was measured and compared to the positive control of the antibiotic (Cephalaxin). A significant inhibition of the two gram-positive species (*S. aureus* and *B. subtilis*). Furthermore, the growth of *E. coli* was affected only by the highest dose of AFEE (40 mg/ml). *P. aeruginosa* was the most resistant species to the bactericidal effects of AFEE. Following these findings, a recent study found that *A. fragrantissima* essential oil has antibacterial action against *Bacillus cereus, E. coli, S. aureus, Aspergillus niger, Penicillium* sp., and *Rhizopus* sp. in tomato media [21]. A previous study discovered that aqueous and hydroethanolic extracts of *A. fragrantissima* aerial parts were ineffective against *E. coli, MRSA, Streptococcus pneumoniae, Enterococcus faecalis, Shigella sonnei, P. aeruginosa, Candida albicans, Candida glabrata*, and *Salmonella typhimurium*, while *Klebsiella pneumonia*, *Bacillus cereus*, and *S. aureus* were more sensitive [26]. Other species in the *Asteraceae* family demonstrated antibacterial activity. The antimicrobial activity of the essential oils of the *Achillea biebersteinii* Afan. from Turkey was effective against *Salmonella enterica serovar typhimirium, S. saureus subsp., Yersinia pseudotuberculosis, Bacillus cereus, Enterobacter aerogenes, B. subtilis* and *Proteus vulgaris* **[27].** The gram-positive bacteria *B. subtilis, B. cereus, S. aureus*, and *Staphylococcus epidermidis* were more susceptible to the essential oil of *A. millefolium* than the gram-negative bacteria S. *typhimurium, Salmonella enteritidis, Salmonella agona*, and *E. coli* [28]. *A. fragrantissima* extracts' antimicrobial action could be attributed to their diverse chemical compositions of fatty acids, terpenoids, and ketones, besides, the sesquiterpene of β-Caryophyllene, which showed antibacterial, antifungal, and antioxidant effects against many gram-positive bacteria [29]. Palmitic and octadecanoic acids (stearic acid), which account for 10% and 6% of the current GC/MS study, respectively, have been shown to exhibit strong antibacterial effects against *P. aeruginosa* and *S. aureus* [30]. Furthermore, caproic acid has a strong antibacterial impact against *Campylobacter jejuni* and *Campylobacter coli* **[31].** All these studies, in

addition to the current findings, suggest the susceptibility of *A. fragrantissima* as a possible antibacterial agent.

AFEE inhibited A594 cell proliferation in a dose-dependent manner, with an IC_{50} of 14.01 µg/mL. These findings are consistent with prior research on *A. fragrantissima* and other *Achillea* species, which have shown strong anticancer activity because of their high bioactive content. Previous studies reported the cytotoxic effect of *A. fragrantissima* against breast cancer cells of MCF-7 [11], pancreatic cancer cells of MiaPaCa-2, prostate cancer cells of PC-3, and lung cancer cells of A594 [7]. Furthermore, the essential oil extract of *A. fragrantissima* demonstrated cytotoxic effects on breast (MCF7) and colorectal cancer (HCT-116) cells, with IC_{50} values ranging from 0.51–0.91 µg/ml [32]. Similarly, *Achillea membranacea* extracts revealed high cytotoxic action against A2780 (ovarian cancer) and HT29 (colon cancer) cells with an IC_{50} of around 12.99 and 14.02 µg/ml, respectively [33]. These findings indicate that distinct *Achillea* species share bioactive chemicals responsible for their anticancer properties, such as flavonoids, sesquiterpenes, and phenolics. Comparable research from Saudi Arabia supports the medicinal herbs' high cytotoxic action against cancer cells. The observed cytotoxicity in A594 cells could be ascribed to the high concentration of flavonoids, terpenoids, and phenolic chemicals in AFEE, which have previously been shown to have anticancer effects. These findings indicate that Saudi Arabian medicinal plants, particularly those in the Asteraceae family, have potent anticancer characteristics that could be further investigated for therapeutic purposes.

AFEE's potential to induce apoptosis was validated by flow cytometry. Treatment with 20 µg/ml increased overall apoptosis to 33.27% compared to 1.44% in the control. Early apoptosis made up 22.85% of the total, with 10.42% proceeding to late apoptosis or full cell death. The highest dose of 40 µg/ml reduced total apoptotic % due to cell debris outside the gated area of the dot plot. This behavior is frequently seen in cells experiencing late-stage apoptosis or necrosis, in which cells shrink, lose integrity, and form apoptotic bodies **[34].** To confirm the processes driving AFEE-induced apoptosis and cell cycle arrest, protein expression analysis was performed. AFEE treatment decreased Bcl-2 expression by 56.14% at 40 µg/ml compared to the control, indicating a dose-dependent effect. In contrast, caspase-3 expression increased by 66.27%, indicating apoptosis. Similar results were found for the aqueous extract of *A. fragrantissima* against breast cancer (MCF7) and HepG2 cells by targeting apoptotic network indicators such as Caspase-3, Caspase-8, Caspase-9, Bcl-2, BAX, and Bcl-xl [35]. On the other hand, other species from the *Asteraceae* family showed similar behavior. The aqueous extract of *Achillea thracica* seeds induced the cytotoxicity of HT1080 fibrosarcoma cells at an IC_{50} of 9.85 g/l **[36].** The hydroethanolic extract of *Achillea millefolium* induced apoptosis in NCI-H460 (lung cancer) and HCT-15 (colorectal cancer), caused an increase in p53 and p21, and reduced XIAP expression levels [37]. The essential oils of *Achillea millefolium* revealed similar findings on the Panc-1 and MCF-7 cancer cell lines [38]. Extracts from other species, including *Achillea membranacea* and *Achillea Wilhelmsii C. Koch*, were also apoptosis inducers against A2780 (ovarian cancer) and HeLa (cervical cancer) cells by altering the LIN28B and p53 genes expression **[12, 34].** These findings demonstrate that AFEE exerts its anticancer

effects by regulating critical apoptotic regulatory proteins, highlighting its potential as a natural cancer treatment.

Cell cycle studies demonstrated that AFEE administration caused considerable G2/M phase arrest in A594 cells. Cells in the G2/M phase rose from 19.18% in untreated control cells to 27.97%, 31.17%, and 36.77% at 10, 20, and 40 µg/ml, respectively. This increase was followed by a significant decrease in the G0/G1 phase population and an increase in sub-G, indicating a high amount of cellular death. Cyclin B levels increased by 74.35% at 40 µg/ml, indicating that AFEE impairs mitotic progression and causes G2/M phase arrest. In comparable studies, *A. fragrantissima* caused cycle arrest through G2-phase cell cycle arrest, which caused the elongation of the human chronic myeloid leukemia (K562) cells **[31].** Also, the 48-hour exposure to the secondary metabolite, rupicoline from *Achillea grandifolia*, induced a G2/M cell cycle arrest of U87MG and T98G Glioblastoma cells [39]. The observed G2/M arrest indicates that AFEE may interfere with cell division by targeting critical mitotic progression regulators, resulting in reduced cancer cell viability.

This study offers a complete phytochemical characterization of *A. fragrantissima*, revealing its rich composition of phenolics, fatty acids, ketones, and terpenoids. It focuses on the plant's powerful antioxidant, antibacterial, and anticancer capabilities, as well as its substantial cytotoxic effects on A594 lung cancer cells. Unlike prior studies that focused on *A. fragrantissima* from other countries, this study looks particularly at specimens produced in Saudi Arabia, providing region-specific insights into its bioactive potential. The findings show that *A. fragrantissima* from Saudi Arabia has high antioxidant and antibacterial activity, implying that the country's particular climatic conditions may influence its chemical composition. These findings lend credence to the plant's prospective pharmacological and nutraceutical applications, notably as a natural alternative to synthetic antioxidants and antibacterial agents. The chemical composition, antioxidant, antibacterial, and anticancer properties of the ethanolic extract from the aerial parts of *Achillea fragrantissima* have important implications for disability research, particularly in the treatment of chronic illnesses, infections, and cancer-related disabilities. Chronic illnesses, including diabetes, arthritis, and cancer, are frequently connected with oxidative stress, which can worsen impairment by destroying cells and tissues. The study emphasizes AFEE's powerful antioxidant action, which may help buffer oxidative stress and improve the quality of life for those with disabilities caused by these illnesses. Furthermore, infections, particularly those produced by antibiotic-resistant bacteria, can result in serious consequences and disability. The study reveals that AFEE has substantial antibacterial action against both gram-positive and gram-negative bacteria, including S. aureus and E. coli. This shows that AFEE may be a natural alternative to infection control, lowering the likelihood of disability caused by severe or recurring infections. On the other hand, cancer and its therapies, such as chemotherapy and radiation, frequently cause physical disabilities owing to tissue damage, discomfort, and functional impairments. The study found that AFEE causes apoptosis and cell cycle arrest in A594 cells, showing its potential as a natural anticancer agent. By targeting cancer cells, AFEE may lessen the need for chemotherapy treatments, which frequently result in impairment, thereby improving patient outcomes.

Conclusions

The findings emphasized *A. fragrantissima*'s potential antibacterial efficacy against harmful bacteria, hinting that its active metabolites may be a hidden source of powerful bioactive compounds. This work successfully characterized the complex phytochemical composition of *A. fragrantissima* from Saudi Arabia, indicating a high phenolic content and powerful bioactive properties. The plant extract exhibits substantial antioxidative, antibacterial, and anticancer activities, indicating its potential utility in both traditional and modern medicine. Its strong cytotoxic activity and induction of apoptosis in lung cancer cells highlight its potential as a natural anticancer medication, prompting further investigation into its therapeutic applications. Given these findings, *A. fragrantissima* from Saudi Arabia could be researched further for pharmaceutical development, particularly for natural antibacterial and anticancer formulations. Future research should analyze the phytochemical content of different regions and conduct in vivo studies to assess the clinical potential.

Materials and Methods

Plant Material and Extract Preparation

A. fragrantissima aerial parts were obtained in March 2023 from the Rafha region of the Kingdom of Saudi Arabia, located at 29°38'19"N 43°30'5"E. The collection and handling of the plant material were according to the International Union for Conservation of Nature (IUCN) Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The plant was deposited in the herbarium of the College of Science, King Saud University, under the name of "*Achillea fragrantissima* shrub, Rafha, Riyadh". Plant identification was performed by Prof. Najat Bukhari in the Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia.

The plant was cleaned with running tap water first, then with sterile distilled water. The plant was then shade-dried, carefully powdered, and stored at 4°C until later usage. The infusion method was used to prepare the plant extract **[40].** The fresh, dry plant components were crushed, and 30 g were soaked overnight in 300 ml of 100% ethyl alcohol in a closed container at room temperature, then transferred to another container overnight using a rotary shaker. The macerates were gravity-filtered twice, once through cotton and then through layers of tissue paper. Finally, the residual filtrates were dried overnight in cleaned metallic trays before being transferred to a clean, dry glass container and refrigerated until needed.

Fourier transform infrared spectroscopy (FTIR)

The functional groups were found using FTIR analysis. A spectrometer (Nicolet 6700, Thermo Fisher Scientific Inc., Waltham, MA, USA) with a beam splitter and detector (DTGS) loaded with OMNIC software was used to gather and analyze spectra in the 500–4000 cm1 scan range. The resulting IR spectra were utilized to analyze the functional groups found in the produced extract. The acquired wavelengths were

evaluated by the Scientific Reaction Animation Image Builder (InstaNANO) https://instanano.com/all/characterization/ftir/ftir-functional-group-search/ to identify the relevant functional groups [41].

Gas Chromatography/Mass Spectrometry (GC/MS)

The ethanol extract underwent GC/MS analysis. The carrier gas was helium, with a flow rate of 1 ml per minute. The Agilent 5977c GC/MSD (Agilent Inc., Santa Clara, California, USA) was used. A phenyl methyl siloxane column (30m, 250m, 0.25m) was utilized. The following settings were maintained throughout the GC run: time (90 minutes), volume (1 L), temperature (280°C; 250°C), ion source, and split ratio (20:1). Furthermore, the temperature was held at 40°C for 5 minutes before increasing to 280°C at 10°C/min and remaining there for another 5 minutes. For mass spectroscopy, an electron impact (70 eV) was created with a scan range of 35 to 780 m/z. The National Institute of Standards and Technology (NIST) mass spectral library (https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:start) was used to identify the samples **[42].**

Scavenging activity assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is widely used to assess the antioxidant activity of plant extracts. This assay evaluates bioactive compounds' ability to scavenge free radicals promptly and consistently [43]. A 0.1 mM DPPH solution in methanol with an initial absorbance of 0.9-1.0 at 517 nm was created. The plant extract or ascorbic acid, a common antioxidant, was produced in methanol at various quantities. Each sample was mixed with an equal volume of DPPH solution before being incubated for 30 minutes in the dark at room temperature. After incubation, the absorbance is measured at 517 nm using a UV-Vis spectrophotometer. The fraction of DPPH scavenging activity is calculated using the following formula:

$$Scavenging\,\% = \frac{Ac - As}{Ac} \times 100$$

Here, “Ac” is the absorbance of DPPH solution alone without any samples, and “As” is the absorbance of DPPH mixed with either the plant extract or the ascorbic acid.

Antimicrobial Activities

The bacterial strains were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These strains included *Staphylococcus aureus* (ATCC-25923), *Bacillus subtilis* (ATCC-6051), *Escherichia coli* (ATCC-11755), and *Pseudomonas aeruginosa* (ATCC-27584). All bacterial strains were cultured on Mueller-Hinton agar medium.

To achieve a concentration of 1 µg/ml, the previously produced AFEE powder was dissolved in a 1:100 DMSO: distilled water solution. Mueller-Hinton agar growth media was made according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 19 grams of Mueller-Hinton agar were dissolved in 500 ml of deionized water before being sterilized in an automated autoclave at 121°C

for 15 minutes. After cooling to 70ºC, each Petri dish received approximately 10 ml of the medium. At room temperature, the medium was allowed to harden. The bacterial strain was plotted on three Petri dishes. Three discs were created, with three concentrations of the extract (10, 20, and 40 µl) inserted in each disc. Another disk was made for the control by adding only 0.2 µl of DMSO. Another Petri dish had cefalexin as a positive control (5 µg/ml). The plates were placed in an incubator at 37°C for 24 hours. After incubation, the areas of growth inhibition were measured in millimeters [44]. All experiments were conducted in duplicate. Minimum inhibitory concentrations (MIC) were determined using the following equation:

$$MIC = \frac{Lowest\ Conc.\ inhibit\ the\ growth + Highest\ conc.\ allow\ the\ growth}{2}$$

Cell culture

In the current study, the lung cancer cell line A594 was used to study the anticancer properties of *A. fragrantissima* ethanolic extract. A594 cell line was obtained from ATCC (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% Glutamax, 10% Fetal Bovine serum, and 1% 10000/1000 penicillin-streptomycin (Thermo Fisher Scientific Inc., GIBCO, Waltham, MA, USA). The cells were incubated in a 37ºC CO_2 incubator, and media were renewed every three days.

Cytotoxicity assay

In this assay, 5000 cells per well were seeded in a 96-well plate and adhered overnight in a humidified incubator at 37°C with 5% CO_2. The following day, cells were treated with varied amounts of the plant extract, while untreated cells were the negative control. After 24 hours of incubation, add 10 µL of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) (Thermo Fisher Scientific Inc., Invitrogen, Waltham, MA, USA) to each well and incubate for 90 minutes. The formazan crystals will form a purple precipitate. The media was carefully removed, and the formazan crystals were solubilized with 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm with a microplate reader [21]. The IC50 was calculated by applying all the resulting optical densities and corresponding concentrations to the IC_{50} Calculator of AAT Bioquest at https://www.aatbio.com/tools/ic50-calculator.

Apoptosis/ Necrosis Assay

The Annexin V/Propidium Iodide (PI) assay was used to identify and distinguish between apoptotic and necrotic cells based on phosphatidylserine externalization and membrane integrity **[45].** Cells were planted into an appropriate culture plate and treated with the plant extract. After 24 hours of incubation, cells were extracted, including adherent and floating populations, and washed with cold PBS. The cells were resuspended in 100 µl of binding buffer at a concentration of 1×10^6 cells/ml. The FITC Annexin V Apoptosis Detection Kit with PI (Biolegend Co., San Diego, CA, USA) was used following the

manufacturer's instructions. The mixture is incubated in the dark for 15 minutes at room temperature before being analyzed using a BD FACSCalibur™ Flow Cytometer (BD Co., Franklin Lakes, NJ, USA).

Cell cycle assay

The PI-based cell cycle test is a popular method for determining DNA content and cell distribution during the cell cycle. Cells were initially treated with the plant extract, then collected and fixed with cold 70% ethanol to retain their structure. After washing to remove any remaining ethanol, the cells were treated with a staining solution containing PI, RNase A to break down RNA, and a detergent to permeabilize the cell membrane. The samples were then analyzed using flow cytometry, with fluorescence intensity used to differentiate between cells in the G0/G1, S, and G2/M phases [46].

Western blotting

To carry out the assay, treated cells were lysed in RIPA buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA) to extract total protein. The protein concentration was determined using the Bradford assay and a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal amounts of protein were separated using SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk to prevent nonspecific binding. It was treated overnight with primary antibodies that targeted apoptosis (Bcl-2 and Caspase 3), cell cycle (Cyclin D1), and the housekeeping protein β-actin (Biolegend Co., San Diego, CA, USA). Following that, the membrane was incubated with suitable HRP-conjugated secondary antibodies and visualized with a c-digit scanner after staining with Luminol and oxidizing solutions (Thermo Fisher Scientific Inc., Waltham, MA, USA). The band intensities were measured by ImageJ densitometry software version 1.8.0_345 https://imagej.net/ij/ **[47].**

Statistical Analysis

The data was statistically evaluated with SPSS version 22. The experiments were subjected to a one-way analysis of variance (ANOVA), and mean differences were analyzed using the Duncan test. At the 0.05 probability level, the mean values were separated using the least significant difference (LSD). All values in this study represent the average of at least three replicates. The data was reported as the mean with standard deviation (SD).

Declarations

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F.A. and T.A.; project administration, F.A.; funding acquisition, F.A. All authors have read and agreed to the published version of the manuscript.

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Figures

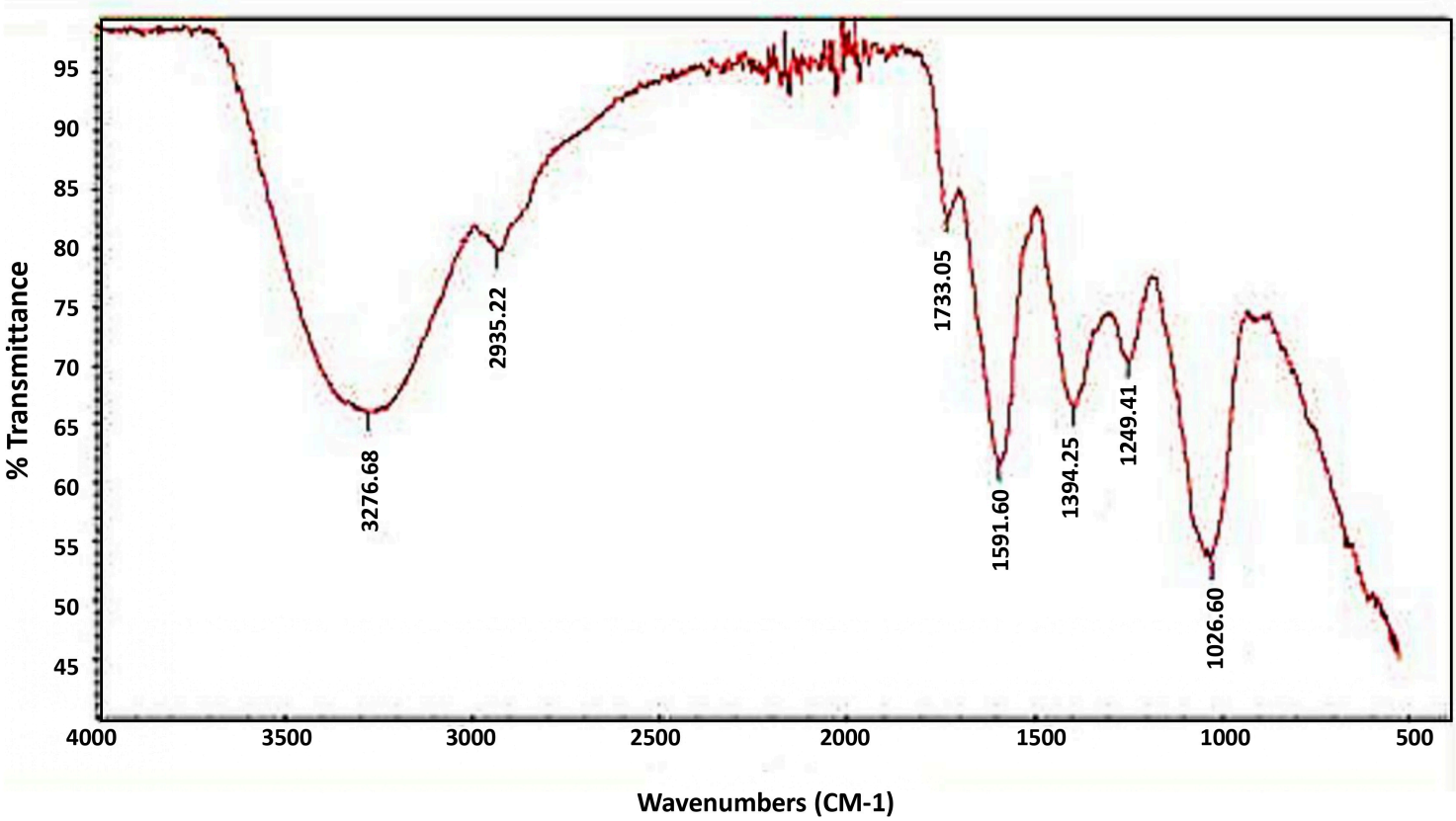

Figure 1

FTIR results of the AFEE. The results were obtained using a Nicolet 6700 FT-IR spectrometer over a range of 4000–500/cm.

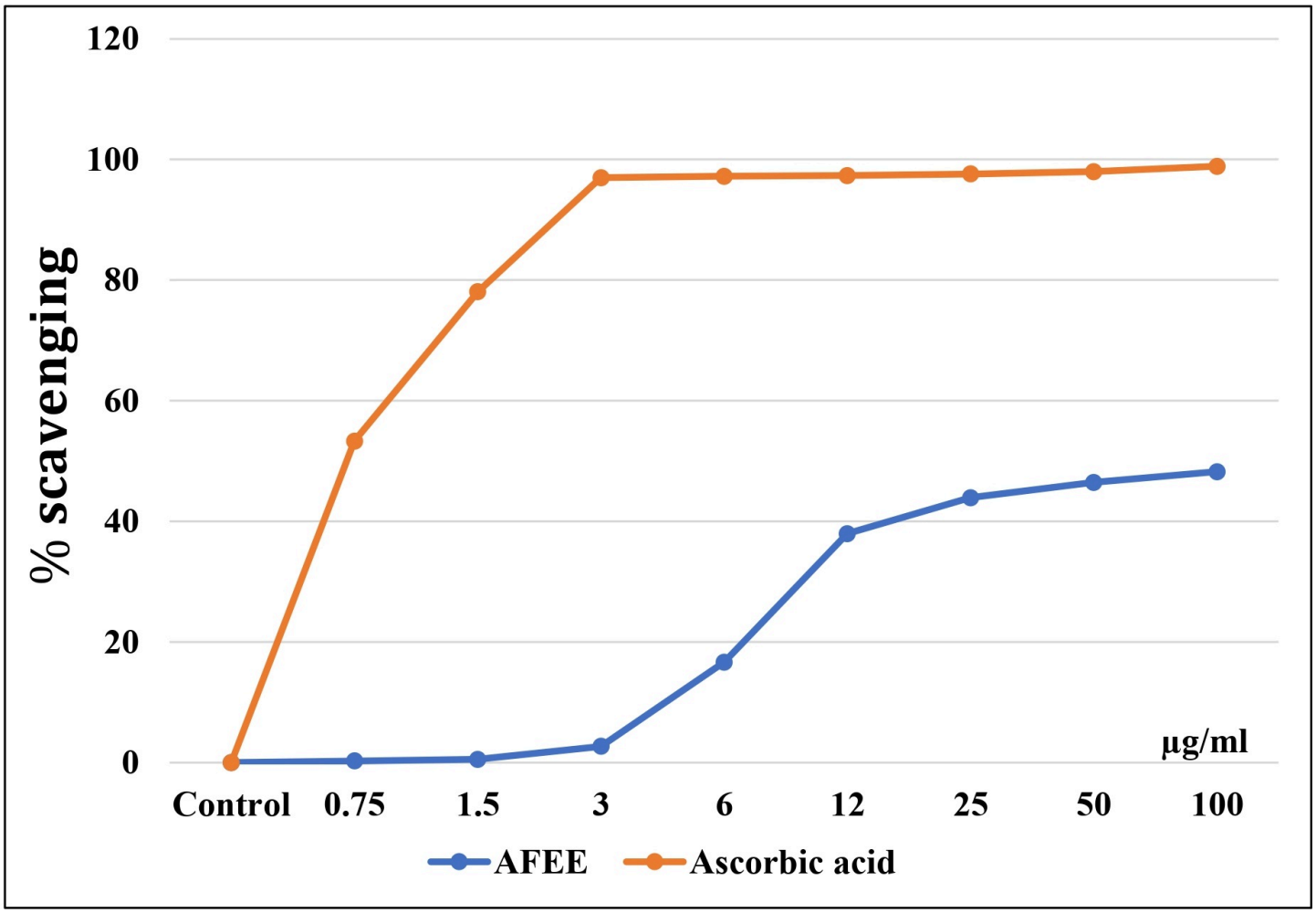

Figure 2

The scavenging activity of AFEE versus ascorbic acid. DPPH assay was used to identify the antioxidant activity. All concentrations from the two materials were measured in triplicate, where the lines with dots express the mean scavenging percentage of all reads.

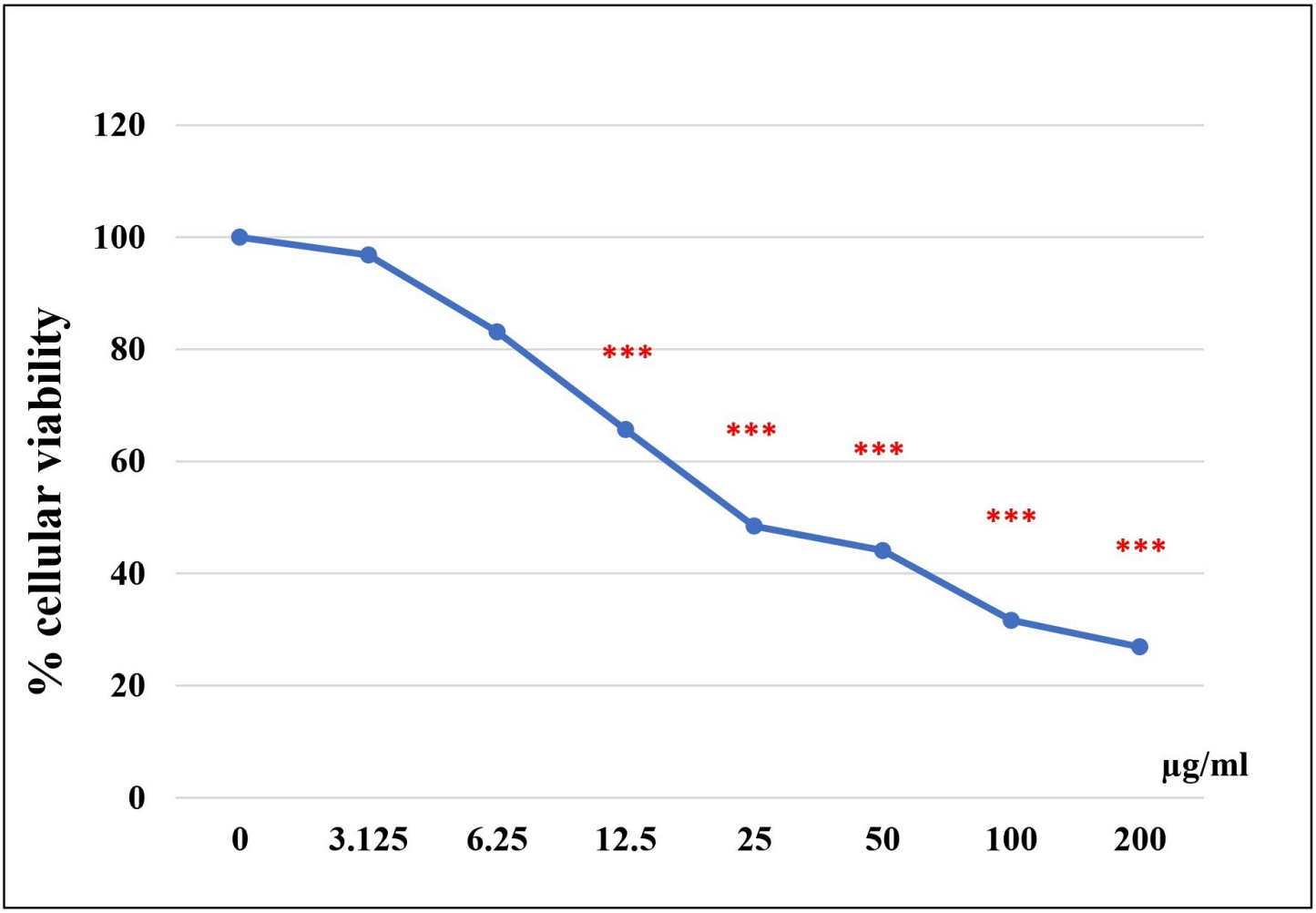

Figure 3

Cytotoxic activity of AFEE against A594 cells. The cancer cells were treated with different concentrations of AFEE (in triplicate) for 24 hours. The MTT assay was used to identify the cytotoxic and antiproliferative activities observed in treated cells compared to the control. *** indicates significant readings at $p < 0.05$.

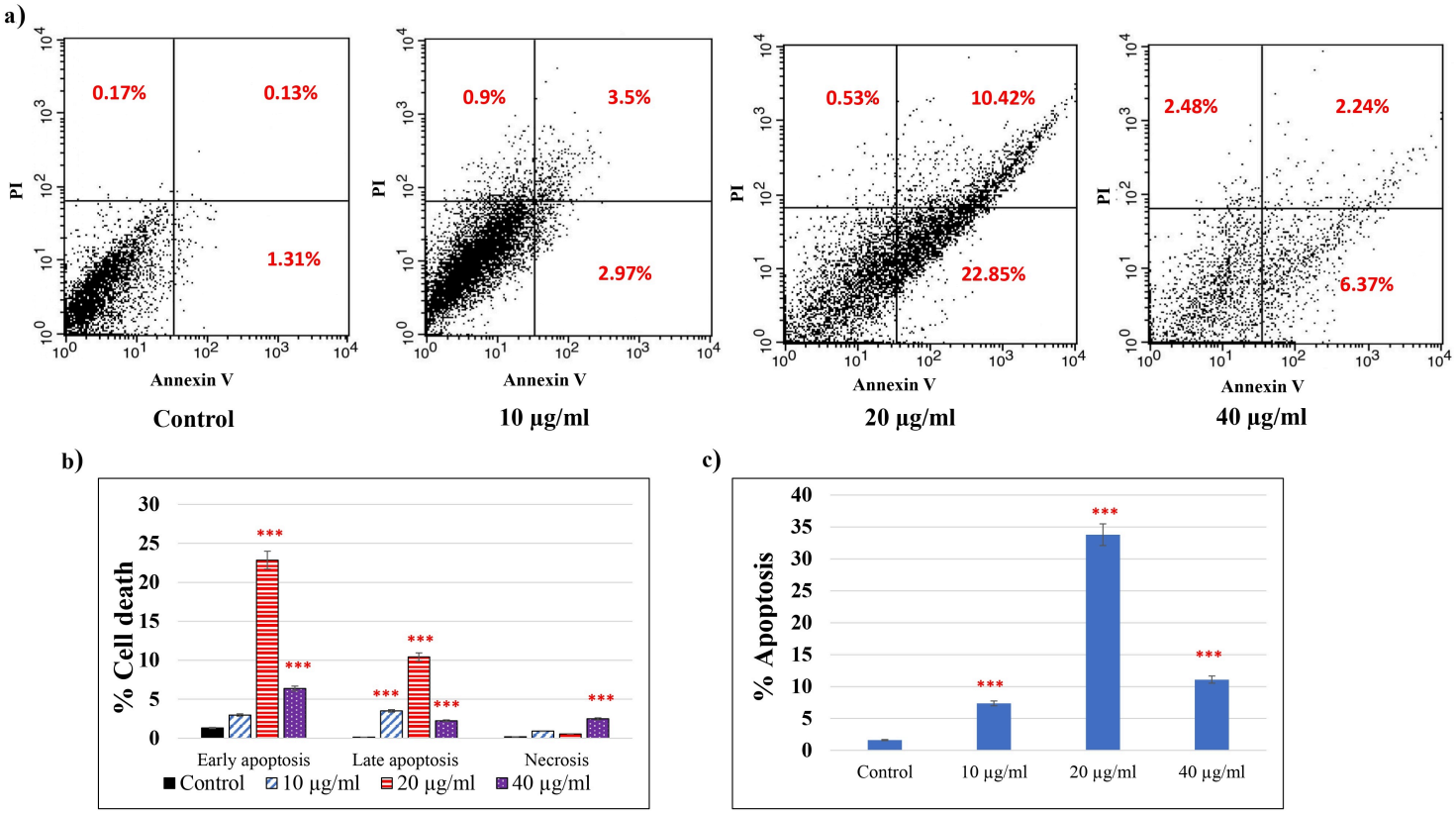

Figure 4

The apoptotic activity of AFEE against A594 cells. The cells were treated with different doses of AFEE for 24 hours, and then the cellular death was detected by Annexin V/Propidium iodide and a Flow cytometer. *** indicates significant readings at p <0.05. a) Dot blots of treated and untreated cells after staining with Annexin V/PI; b) Different cell death structures observed from the dot blots at all concentrations. c) the total apoptotic effect (early + late apoptosis) at all concentrations.

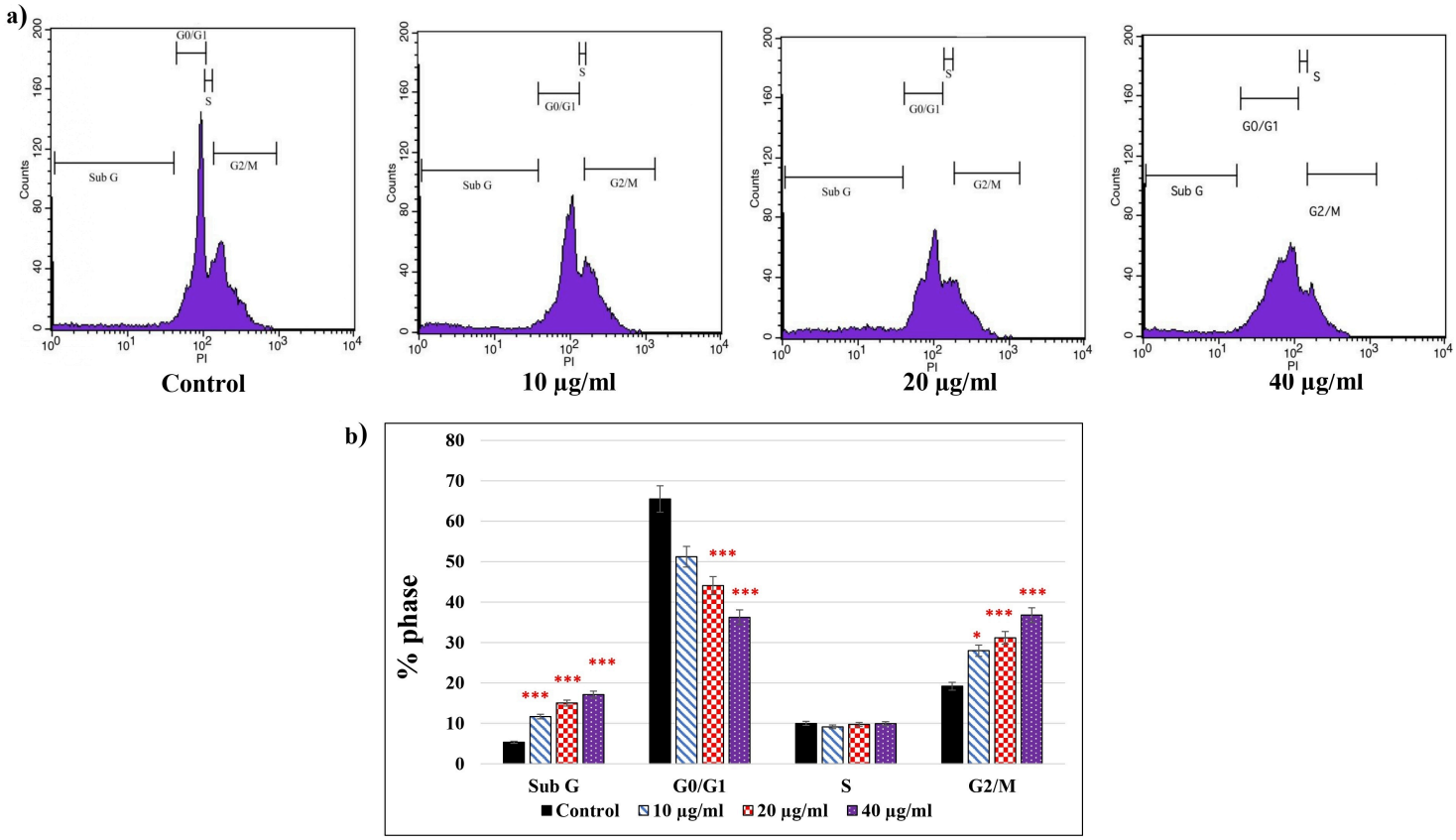

Figure 5

The Cell cycle analysis of A594 treated with different doses of AFEE. The cells were treated with different doses of AFEE for 24 hours, and then the cell cycle was analyzed using Propidium iodide and a Flow cytometer. *** indicates significant readings at p <0.05. a) Histograms of treated and untreated cells after staining with PI; b) Different cell cycles expressed in treated cells and compared to the control cells.

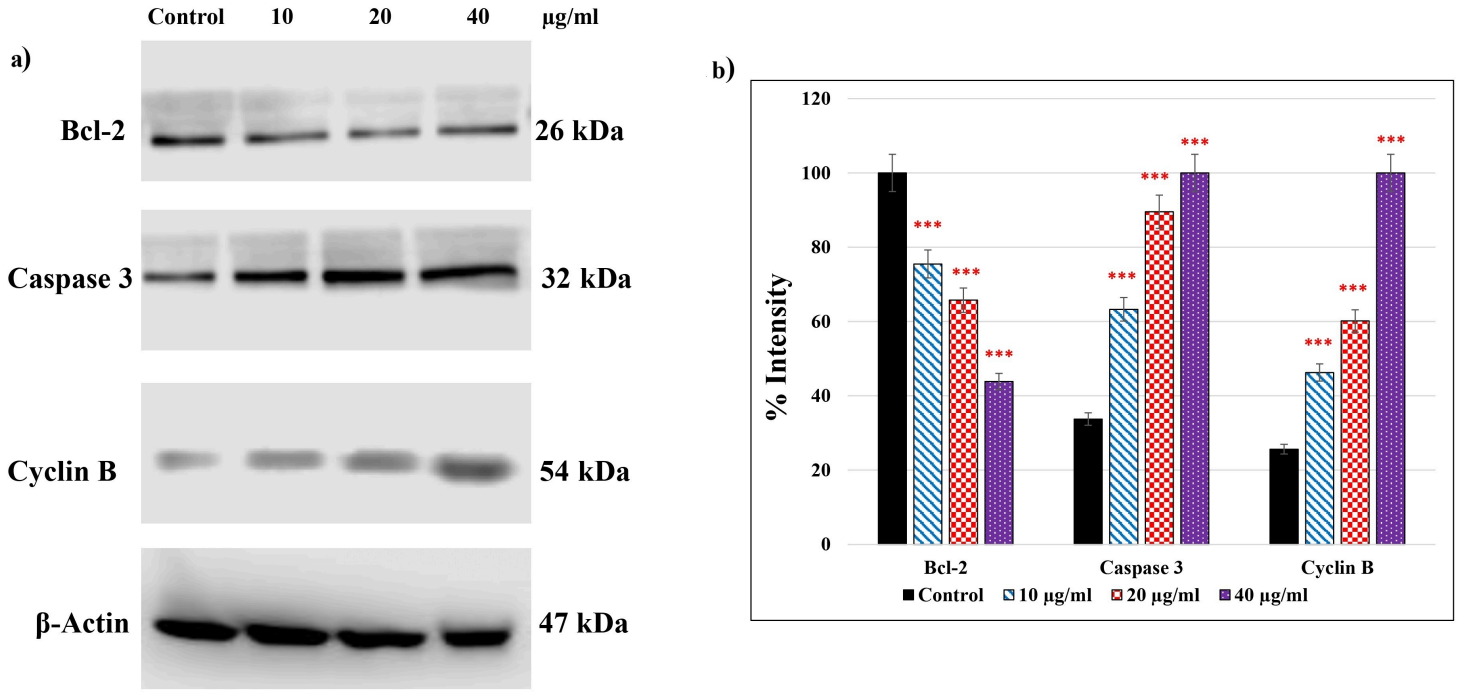

Figure 6

The anticancer activity of AFEE against the cellular apoptotic and cellular arrest protein markers. The cells were treated with different doses of AFEE for 24 hours, and then western blotting was used to detect the apoptotic pathway proteins' expression of p53 and caspase 3, in addition to cyclin B as an indicator of G2/M phase arrest. *** indicates significant readings at p <0.05. a) Immunoblots of AFEE-treated and untreated cells compared to the housekeeping protein of β-actin; b) Band intensities calculated for all proteins and normalized to β-actin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- gelimages.pdf