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Research Article

Insight into possible therapeutic applications of *Ephedra transitoria*: *in-vitro* biological, toxicological activities and GC-MS analysis of aerial parts' extract

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Abstract

Most Arab nations have arid regions where *Ephedra transitoria*, a plant, flourishes. It has a variety of medical benefits. It belongs to the Ephedraceae family. The plant's aerial portions were procured from the Hail region of Saudi Arabia, methanol was used to extract bioactive phytochemicals with variable solubility in an efficient manner, and the resulting extract was tested on several cancer cell lines. The extract's cytotoxic potential was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay method against standard vinblastine sulfate and for each cell line, the pertinent half maximal inhibitory concentration values were calculated. Additional apoptosis assays were conducted. Also, the antioxidant activity of plant extract was examined using 2, 2-Diphenyl-1-picrylhydrazyl and [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] methods compared to ascorbic acid standard. Also, antimicrobial effect of extract was evaluated on different gram positive and gram-negative bacteria by zone inhibition and minimum inhibitory concentration assay methods. GC-MS analysis was further applied to identify major bioactive substances. Extract showed good cytotoxic activity against both lung and liver cancer cells with relevant half maximal inhibitory concentration values comparable to those of standard. Extract resulted in S-phase arrest, primarily via inducing apoptosis mediated through activating caspase-3, Bax, and p53 proteins. Plant extract showed good antibacterial activities against *S. aureus, E. coli* and *B. subtilis*. Also, it showed high antioxidant activity with relevant half maximal inhibitory concentration values comparable to those of standard. Some bioactive compounds were separated and identified by GC-MS analysis such as; ephedrine and quercetin. *ephedra transitoria* extract possess high cytotoxic activity against lung and liver cancer cells via S-phase arrest mediated by activation of apoptotic proteins. It also possesses high antioxidant and antibacterial activities.

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Keywords: antibacterial; antioxidant; apoptosis; cytotoxic; *Ephedra*

Abbreviations: **A549** stands for human lung cancer**;** **ABTS** stands for 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **DPPH** stands for 2, 2-Diphenyl-1-picrylhydrazyl; **HCT116** stands for human colorectal cancer**;** **HepG2** stands for human hepatocellular carcinoma**;** **IC_{50}** stands for half maximal inhibitory concentration; **MCF7** stands for human breast cancer; **MIC** stands for minimum inhibitory concentration; **MTT** stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.

Introduction

Ephedra is a member of the Ephedraceae family, which is mostly found in dry and semi-dry settings. Syria to the Arabian Peninsula is where this species formerly lived. It is a subshrub that thrives mostly in deserts. In the traditional medicine of many different nations, several species in this genus are frequently used to treat asthma, the common cold, the flu, chills, fever, headache, nasal congestion, and cough. Due to the pharmacological properties of the ephedrine-type alkaloids they contain, *Ephedra* species have had their chemical makeup researched for decades (González-Juárez *et al*., 2020).

The various chemical components of the species, such as phenolic and amino acid derivatives, have also provided evidence for the ethno-medical usage of Ephedra species. The subtropics and tropics' drier regions are home to the genus *Ephedra* L. (González *et al*., 2020) which contains more than 69 species. *Ephedra alata*, *Ephedra transitoria*, *Ephedra foliata* and *Ephedra alteboissis* are the four most prevalent of them (Hollander *et al*., 2010).

The two most frequently researched active ingredients in ephedra, (-)-ephedrine (Wong *et al*., 2015) and (+)-pseudoephedrine (Calzada *et al*., 2020), which have been isolated from the plant's seeds, flowers, leaves, and roots, have been linked to a variety of pharmacological properties, including those for the treatment of diabetes, obesity, and inflammatory diseases. Tachycardia, anxiety, nausea, headaches, dizziness, and an elevated risk of myocardial infarction, stroke, and unexpected death are just a few of the significant side effects that could occur. Despite the fact that pharmacological testing on the majority of the isolated chemicals from the numerous *Ephedra* species is lacking, the data that is available suggests that they have great promise as a source of natural products with medicinal, aesthetic, nutritional, and agro-industrial application. (Wooltorton and Sibbald, 2002).

Notable research gaps include those in the photochemistry and assessment of the pharmacological properties of Ephedra species, in particular. This has led to the hypothesis that they either contain very little, if any, ephedrine-type alkaloids, or none at all. Additionally, the non-alkaloidal compounds of the Ephedra genus exhibit noteworthy biological activity potential and may be physically altered to enhance existing activities or employed as models or scaffolds for the synthesis of novel molecules with physiological activity (González-Juárez *et al*., 2020**).** Finally, study into the endophytic fungal strains linked with Ephedra species has significant benefits for the chemical, ecological, microbiological, and medicinal domains (Bashyal *et al*., 2005)

Due to its historical use as an appetite suppressant (Song *et al*., 2012), asthma treatment (Stewart *et al*., 1929), diabetes treatment (Meduru *et al*., 2016), wound healing impact (Kittana *et al*., 2017), anti-inflammatory activity (Liang *et al*., 2018), cytotoxic and anti-tumor activities (Mendelovich *et al*., 2017) and antiviral activity (Guo *et al*., 2006), several species have been researched for their ethnobotanical usefulness. In order to pave the way toward prospective areas for additional research, this paper gives a current knowledge foundation on *Ephedra transitoria*, including its phyto-chemistry and pharmacological properties such as antiviral activity. In the present work, our aim is to evaluate the biological activities of *Ephedra transitoria* methanolic extracts of aerial parts, such as antioxidant, antimicrobial and their anticancer capabilities in

addition to separation and identification of some bioactive substances by GC-MS analysis technique. These results can be further applied in formulation of new drugs such as antibacterial and anticancer ones.

Materials and Methods

Chemicals and solvents

All chemicals and solvents used were of high quality and analytical grade from Sigma-Aldrich (St. Louis, MO, USA)

Collection and extraction of plant material

In the Saudi Arabian province of Hail, *Ephedra transitoria* was discovered, recognized, and inside the pharmaceutical chemistry department was then kept a sample of it. The air dried powdered aerial parts (about 1 kg) were extracted by repeated cold maceration with 70% methanol (3 x 5 Liters) to allow extraction of bioactive phytochemicals with variable solubility. The methanolic extract was combined and further concentrated under vacuum to give about 75 g of residue of aerial parts crude extract (EST).

GC-MS (Gas Chromatography-Mass Spectrometry) analysis

Using GC-MS methods, we conducted additional research on methanolic extracts. Thermo MS-DSQ II and 5.0 Thermo GC-TRACE (Thermo Scientific, Austin, TX, USA) extreme versions of equipment are available from Thermo Scientific Co. DB-5 Non-polar Capillary column, 30Mts, ID of 0.25 mm thickness, were found to be the optimal chromatographic conditions. 1 ml/min was chosen as the flow rate. The temperature was intended to rise at a rate of 6 °C/min while using a 1l injection volume over a temperature range of 70 to 260 °C. Utilizing a Thermo Scientific Trace GC-TSQ mass spectrometer from Thermo Scientific, Austin, TX, USA and a capillary column TG-5MS with dimensions of 30 m x 0.25 mm x 0.25 m film thickness, methanolic extracts were chemically evaluated. The temperature of the column oven was started at 50 °C, increased at a rate of 5 °C/min to 250 °C, and then held steady for 2 min. At a rate of 30°C per minute, the temperature was then increased to 300 °C and remained there for two minutes. The MS transfer line was maintained at 260 °C, while the injection unit was kept at 270 °C. Throughout the test, helium was employed as the carrier gas at a constant flow rate of 1 ml/min.

Biological activities

Antioxidant assay

Using the 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS free radical scavenging assay (Cano *et al*., 2023) and the (2,2-Diphenyl-1-picrylhydrazyl) DPPH free radical scavenging assay (Yen *et al*., 1995), researchers evaluated the antioxidant activity of plant extract through measurement in decrease of absorbance by double beam spectrophotometer (Unicam Helios Alpha, United Kingdom) at 734 and 517 nm, respectively. The average values were taken into account.

ABTS free radical scavenging assay

The antioxidant qualities of the extract were also evaluated using the ABTS free radical scavenging method. The ABTS radical cation (ABTS+) in combination with diluted plant extract is measured spectrophotometrically at 734 nm using double beam spectrophotometer (Unicam Helios Alpha, United Kingdom) (Cano *et al*., 2023) using ascorbic acid and methanol as the standard antioxidant and the unfavorable control. The amount of ABTS radical scavenging was calculated using the formula below:

Impairment (%) = 100 (Control absorbance minus extract absorbance) Consumption of control

DPPH free radical scavenging assay

The DPPH assay was used to gauge the extract's antioxidant potential. To assess the activity of the extract at various doses with, ascorbic acid was utilized as a positive control (Yen *et al*., 1995). At 517 nm, color loss was quantified spectrophotometrically by double beam spectrophotometer (Unicam Helios Alpha, United Kingdom) Following is how the proportion of DPPH radical scavenging was determined: Impairment (%) is calculated as follows: 100 (Absorbance extract - blank) / (Absorbance extract - blank).

Antimicrobial activity

Bacteria and fungi

Various Gram-positive and Gram-negative bacteria, as well as yeast, were employed to assess the antibacterial abilities of *Ephedra transitoria*. Due to their potential to contaminate food and infect humans, the pathogenic bacteria were selected for analysis based on their relevance. Bacterial and fungal strains were obtained from bacteria and fungi stock present in the regional center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Bacterial strains used are *Staphylococcus aureus* (ATCC 25933), *Bacillus subtilis* (NRRL B-543), *Escherichia coli* (ATCC 25932), and *Proteus vulgaris* (ATCC 13326). *Aspergillus fumigates* (RCMB 002008) and *Candida albicans* (ATCC 10231) are two fungi strains. All bacterial and fungal strains underwent a 24-hour incubation period at 37 °C.

In-vitro antibacterial / antifungal bioassay

Using the previously described agar well diffusion method, the antibacterial and antifungal activity of the methanolic extracts was evaluated. (Abo-Ashour *et al*., 2018; Latif *et al*., 2014).

The test microorganism was grown in a volume of 100 mL using 10 mL of new medium. The media attained a count of approximately 108 cells/mL for bacteria or 105 cells/mL for fungi. Following inoculation into agar plates, agar gel was cut into 6mm holes, and 100 mL of each sample were then added to each well. The plates were incubated for 24-48 hours at 37 °C and for 48 hours at 28 °C for bacteria, yeast, and filamentous fungus. Microbial growth was then observed. The inhibitory zone widths around each extract were measured in millimeters, and the outcomes were compared.

Broth micro-dilution method for MIC determination

Different amounts of methanol extract were investigated for their capacity to inhibit bacterial growth. The minimum inhibitory concentration (MIC) of the methanol extract was determined in triplicate for each of the tested bacterial species. The lowest concentration that fully prevented growth in the sample was identified as its MIC during incubation. (Rajendrasozhan *et al*., 2021).

Toxicity studies

Cancer cell lines

MCF-7 cells (ATCC No. HTB-24™-human breast cancer cell lines), HepG-2 cells (ATCC No. HB-8066™ hepatocellular carcinoma cell lines), HCT-116 cells (ATCC No. CCL-248™ Colon carcinoma cell lines) and A-549 cells (ATCC No. CCL-188™-human lung carcinoma) were purchased from American type culture collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI-1640 media that also contained 10% fetal calf serum that has been inactivated and 50 g/ml gentamycin. The cells were sub-cultured two to three times each week while being incubated at 37 °C in a humid environment with 5% CO_2.

Cell viability assay

At a density of 5×10^4 cells/well, the tumor cell lines were spread out on Corning® 96-well tissue culture plates. The plates were incubated for 24 hours. The extracts were then mixed to make a total of twelve concentrations for each ingredient using three copies of each extract. Each 96-well plate that was used as a

control received either medium or 0.5% DMSO. The MTT test was used to determine the quantity of viable cells following a 24-hour incubation period (Salam *et al*., 2022).

Apoptosis analysis (Annexin V-FITC assay) of A549 cells

Using flow cytometry and Annexin V-FITC kits, analyze apoptosis in A549 (lung cancer) cells. Apoptotic cells were further evaluated using the annexin V-FITC assay. According to instructions, the tested extract was used on the grown A549 (lung cancer cell lines) at the IC_{50} concentration. After 72-hour treatment period, A549 cells were removed, rinsed twice in PBS for 20 minutes each, and then rinsed with binding buffer. Additionally, 1 mL of FITC-Annexin V was added to 100 mL of kit binding buffer that included suspended cells. After that, it was incubated for 40 minutes at 4 °C. Following a wash, cells were re-suspended in 150 mL of binding buffer that contained 1 mL of DAPI (1 g/mL in PBS) and were then added to the buffer (Eldehna *et al*., 2018).

Cell cycle analysis using flow cytometry

The A-549 cell line's cell cycle distribution was analyzed using cell cycle analysis in accordance with the previously mentioned protocols to determine the impact of the tested drug on that cell line's cell cycle distribution. The additional material section contains information about the flow cytometry-based cell cycle study.

Statistical analysis

All measurements were carried out in triplicate to get statistically significant data. The results were expressed as an average value (mean) ± standard deviation (SD) at $P < 0.05$ using Graph Pad prism statistical software version 7.

Results and Discussion

The GC-MS chromatogram of methanolic extract of *Ephedra transitoria* showed 17 peaks. These peaks might be connected to bioactive compounds. These peaks were further identified by comparing the mass spectral fragmentation patterns of these peaks with those of the established standards. Six compounds were found as shown in Figure 1.

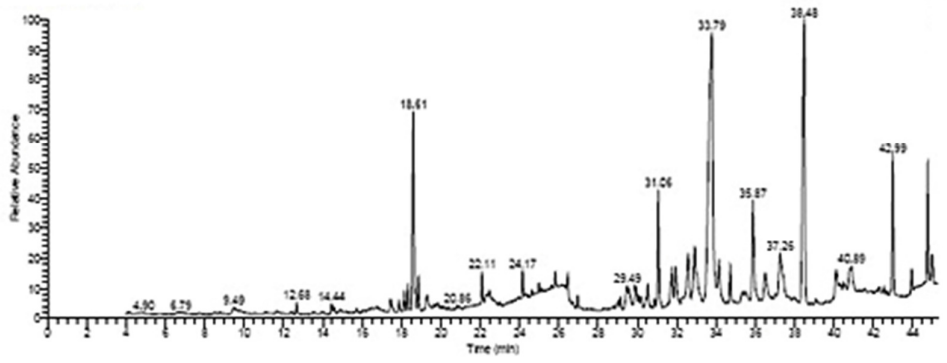

Figure 1. GC-MS Chromatogram for EST extract

According to Table 1, the main phytochemical components were epicatechin, megastigmatrienone, cedrol, δ-Sitosterol, ephedrine and quercetin. Epicatechin is a biologically active substance that plays important roles in the health of cardiovascular and central nervous system (Prakash *et al*., 2018). Megastigmatrienone is a main component of terpenes that play an important role in decreasing cancer cell growth (Kyslychenko *et al*., 2010). Cedrol is a natural sesquiterpene substance that has antibacterial (Ikhoon *et al*., 2011) and antitumor activities, especially against colorectal cancer (Chien *et al*., 2022). Ephedrine is an alkaloid that possess antipyretic and diaphoretic effects (Tang *et al*., 2023). δ-Sitosterol is a chemical substance that interfere with cell proliferation, survival and cell cycle apoptosis that induce anticancer effect (Khan *et al*., 2022). Quercetin is a potent antioxidant flavonoid that protects cells from damage by free radicals (David *et al*., 2016). Moreover, other bioactive substances were identified such as; heptadecane, docosane, *p*-allylansiole, methylephedrine, transtorine, tricosane, bis (2-ethyl) phthalate, 7-octen-2-ol, 5-(benzoyloxy) pentanal, citronellol and ephedradine A. Heptadecane is a straight chain alkane component that possess potential biological effects such as antibacterial activity (Naeim *et al*., 2020). Docosane is a hydrocarbon compound that has an important antimicrobial activity (Sukatar *et al*., 2006). P (4-) allylanisole is an important chemical constituent of many aromatic plants that possess anti-inflammatory effect (Parveen *et al*., 2023). Methylephedrine is a natural component that possess different medicinal effects such as, analgesic, antibacterial and anti-inflammatory (Sajeesh and Parimelazahagan, 2014). Transtorine is a natural quinolone alkaloid that has antibacterial activity against wide spectrum of microorganisms (Al-Khalil *et al*., 1998). Bis (2-ethylhexyl) phthalate is an organic compound that has a role as an apoptosis inhibitor (Cruz-Ramirez *et al*., 2021).

Table 1. The major chemical composition of EST aerial parts' extract

Extract	Compound Name	M.Wt	RT	Area %	Biological activity
EST	Epicatechin	290	31.06	4.65	Cells regeneration effect (Prakash *et al.,* 2018)
	Megastigmatrienone	190	18.86	1.27	Anti-tumor effect (Kyslychenko *et al.,* 2010)
	Cedrol	222	18.33	1.01	Antibacterial and anti-tumor effects (Ikhoon *et al*., 2011; Chien *et al*., 2022)
	Ephedrine	165	21.06	4.36	CNS stimulant (Tang *et al*., 2023)
	δ-Sitosterol	414	44.76	4.28	Anti-cancer effect (Khan *et al*., 2022)
	Quercetin	302	42.99	5.39	Anti-oxidant and antibacterial effect (David *et al*., 2016)
	Heptadecane	240.5	24.17	0.88	Antimicrobial effects (Naeim *et al*., 2020)
	Docosane	310.6	22.11	1.15	Antimicrobial effect (Sukatar *et al*., 2006)
	p-Allylanisole	148.2	29.48	1.08	Anti-inflammatory effects (Parveen *et al*., 2023)
	Methylephedrine	179.3	31.75	1.73	Analgesic, antibacterial and anti-inflammatory effect (Sajeesh and Parimelazahagan, 2014)
	Transtorine	189.2	32.58	2.17	Antibacterial effect (Al-Khalil *et al*., 1998)
	Tricosane	324.6	34.15	1.51	Antibacterial effect (Samadi *et al*., 2012)

	Bis (2-ethylhexyl) phthalate	390.6	36.49	1.26	Apoptosis inhibitor (anti-tumor effect)- (Cruz-Ramirez *et al*., 2021)
	1-Octen-3-ol	156.3	17.47	1.7	Antifungal effect (Wang *et al*., 2022)
	5-(benzoyloxy)-pentanal	192.3	19.32	0.75	Antifungal effect
	Citronellol	156.3	26.46	0.83	Analgesic and anti-inflammatory effects (Santos *et al*., 2019)
	Ephedradine A	492.6	40.11	1.28	Hypotensive effect (Hikino *et al*., 1983)

Biological activities

ABTS scavenging assay of EST Methanolic extract

The ABTS scavenging assay, a decolorization technique, evaluates the antioxidant capacity by a reaction with ABTS radicals (Miller *et al*., 1993). Following a process outlined by others, antioxidant activity in the ABTS scavenging method was assessed (Ling *et al*., 2009).

The ABTS technique is commonly utilized to ascertain the capacity of various antioxidant compounds to scavenge free radicals. Because antioxidant agents have the ability to donate electrons or hydrogen, ABTS has the ability to scavenge. Antioxidant activity has risen in a dose-dependent way, according to research on ABTS results (Ahmeda *et al*., 2020). The results obtained, which are listed in Table 2, demonstrated that both approaches work in tandem and are dependent on the extract concentration and incubation period. From ABTS assay results, it was found that dose response profile of scavenging activities was observed over a dose range from 0 - 30 µg/ml for EST extract. Mean minimal inhibitory concentration IC_{50} was calculated and it was found that EST extract possess high antioxidant activity (with IC_{50} of 14.84 µg/ml). This activity is comparable to that of standard ascorbic acid (with IC_{50} of 2.74 ± µg/ml), as shown in Table 2.

DPPH scavenging assay of EST methanolic extract

Based on the ability to donate hydrogen or scavenge radicals, the DPPH approach was applied to the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical (Choi *et al*., 2002). Antioxidant activity is measured by % decrease in absorbance of DPPH radical. According to the results of DPPH experiment, it was found that dose response profile of scavenging activities was observed over a dose range from 0 - 35 µg/ml for EST extract. Mean minimal inhibitory concentration IC_{50} was calculated and it was found that EST extract has high antioxidant properties (with an IC_{50} of 17.39 µg/ml). This activity is comparable to that of standard ascorbic acid (with an IC_{50} of 6.49 µg/ml), as shown in Table 2.

Table 2. IC_{50} values of EST aerial parts' extract for antioxidant activity by ABTS and DPPH scavenging methods

	Concentration (µg/ml)	% inhibition of ABTS	IC_{50} (µg/ml)	% inhibition of DPPH	IC_{50} (µg/ml)
EST Extract	5	$13.71^{a} \pm 1.53$	14.84	$11.60^{i} \pm 1.43$	17.39
	10	$26.42^{b} \pm 1.44$		$23.32^{k} \pm 1.19$	
	15	$50.16^{j} \pm 1.05$		$46.15^{n} \pm 1.35$	
	20	$63.22^{d} \pm 1.32$		$55.12^{o} \pm 1.41$	
	40	$92.11^{f} \pm 1.70$		$89.42^{p} \pm 1.62$	
Ascorbic acid	1	$13.66^{a} \pm 1.18$	2.74	$10.76^{i} \pm 1.28$	6.49
	2	$37.78^{c} \pm 1.24$		$19.65^{m} \pm 1.33$	
	4	$62.04^{d} \pm 1.38$		$37.51^{s} \pm 1.42$	
	6	$74.31^{h} \pm 1.15$		$48.61^{t} \pm 1.25$	
	10	$91.55^{f} \pm 1.64$		$87.78^{p} \pm 1.54$	

Mean in the same column followed by the same letter are not significantly different at ($P<0.05$)

Antimicrobial activity of EST methanolic extract

In particular when there is drug resistance, natural products contain a variety of chemicals that could be employed as novel antibacterial treatments. Many secondary metabolites that have been isolated from therapeutic plants may have antibacterial properties that help decrease resistance (Kalayou *et al.*, 2012). Investigations into the *Ephedra transitoria* pathogen's antibacterial activity was made against a variety of pathogens. EST Methanolic extract was tested using the qualitative disc diffusion method against a variety of gram-positive bacteria, including *S. aureus* and *B. subtilis*, gram-negative bacteria, including *E. coli* and *P. vulgaris*, and fungi, including *A. fumigatus* and *C. albicans*.

Figure 2 provides summaries of the outcomes. Results showed that the methanolic extract of aerial parts exerted good antimicrobial activity against *S. aureus, B. subtilis, E. coli and P. vulgaris* with an inhibition zone of 17, 15, 16 and 12 mm, respectively for *S. aureus, B. subtilis, E. coli and P. vulgaris*

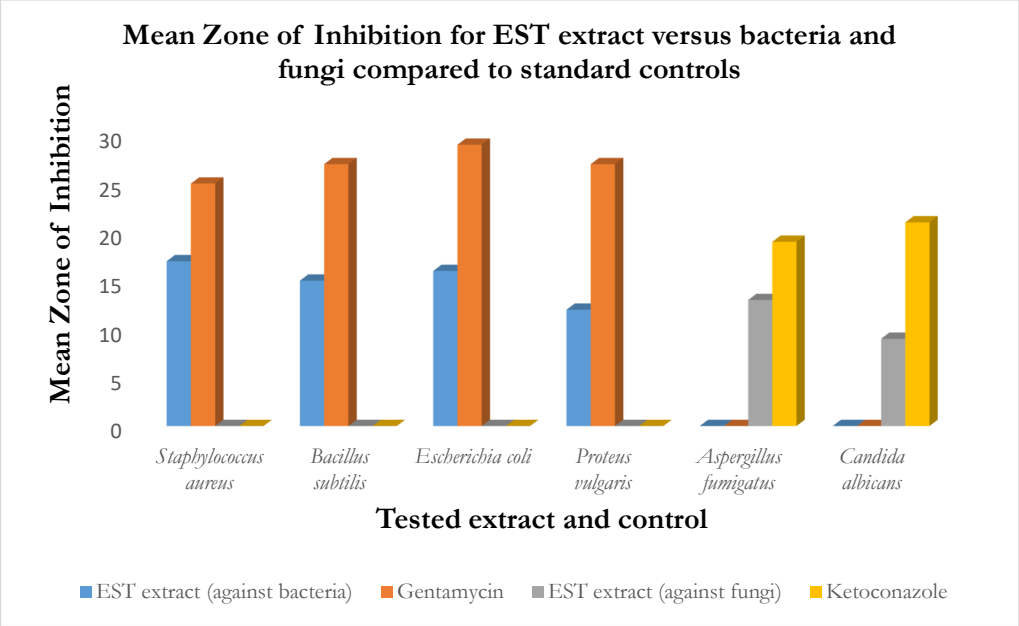

Figure 2. Mean zone of inhibition for EST extract and standards gentamycin and ketoconazole versus bacteria and fungi

Further quantitative evaluation of the extract's antibacterial activity using a broth micro-dilution test investigated the minimum inhibitory concentration (MIC) of the extract against microorganisms. The test medium for the bacterial strains was Mueller-Hinton broth. 100 µL of microbial suspensions (bacteria) were injected with varying concentrations of the extracts in a 96-well microtiter plate. The extract's lowest concentration—which did not result in any discernible growth of the tested organisms—was determined to be the minimum inhibitory concentration (MIC) after a 24-hour incubation period at 37 °C (Rajendrasozhan *et al.*, 2021). Results of the assay corroborated those of the disc diffusion assay, whereby EST extract was found to be most active against *S. aureus and with moderate activity against B. subtilis and E. coli* with MIC values of 156.25, 625 and 625 µg/ml, respectively (as shown in Table 3).

Table 3. The antimicrobial / antifungal activity as minimum inhibitory concentration (MIC) in (µg/ml) of tested microorganisms

Tested Microorganism	Sample name	Control
Fungi	EST	Ketoconazole
Aspergillus fumigatus (RCMB 002019)	650	19
Candida albicans (ATCC 10244)	750	21
Gram Positive Bacteria	EST	Gentamycin
Staphylococcus aureus (ATCC 25933)	156.25	9.7
Bacillus subtilis (NRRL B-543)	312.5	5.2
Gram Negative Bacteria	EST	Gentamycin
Escherichia coli (ATCC 25932)	625	3.6
Proteus vulgaris (ATCC 13326)	1250	4.8

*NA: No activity. The test was done using Micro-dilution method in Micro-titre plate

Cytotoxicity studies

As illustrated in Figure 3, the methanolic extract of *Ephedra transitoria* aerial parts was evaluated against HepG2, A549, HCT-116, and MCF7 cancer cell lines. Table 4 summarizes the IC_{50} values that were obtained.

It was discovered that EST extract exhibits potent cytotoxic activity against both the A-549 and HepG2 cell lines, with an IC_{50} of 19.5 and 21.8 µg/ml, respectively. While it has weak cytotoxic action against HCT-116 and MCF7 cancer cell lines, this activity is comparable to that of standard vinblastine sulfate (with IC_{50} values of 1.14 ± 0.43 and 0.89 ± 0.13 µg/ml for A-549 and HepG2 cell lines, respectively), as shown in Table 4.

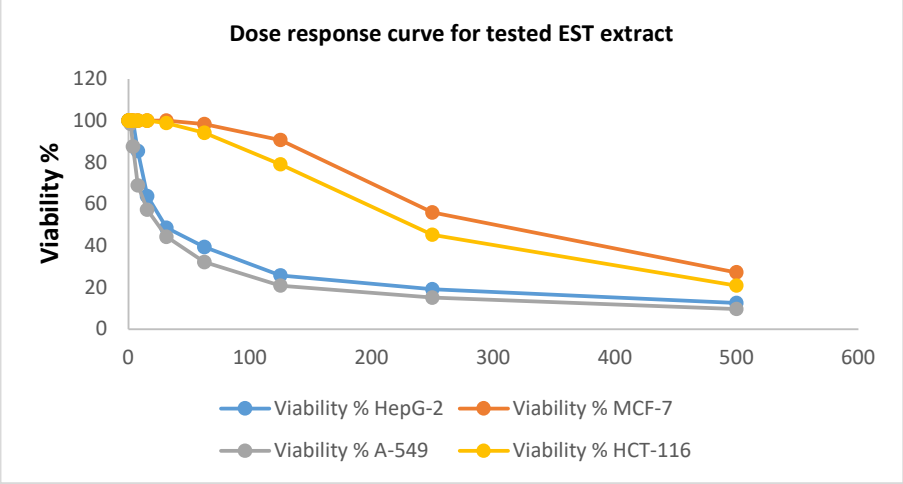

Figure 3. Dose response curves indicating cytotoxic activity of *Ephedra Transitoria* (EST) extract on the viability of HepG2, MCF-7, A-549, HCT-116 cancer cell lines

Table 4. IC_{50} values of EST aerial parts extract against HepG2 andA-549, cancer cells

		HepG2 cells		A-549 cells	
Treatment	Concentration (µg/ml)	% of Dead Cells	IC_{50} (µg/ml)	% of Dead cells	IC_{50} (µg/ml)
Control	0	0	0	0	0
EST Extract	10	$24.81^{a} \pm 1.63$	21.8	$26.10^{i} \pm 1.53$	19.5
	15	$36.52^{b} \pm 1.54$		$37.32^{k} \pm 1.29$	
	20	$49.16^{j} \pm 1.15$		$51.17^{n} \pm 1.25$	
	40	$66.42^{d} \pm 1.52$		$75.22^{o} \pm 1.31$	
	60	$91.82^{f} \pm 1.80$		$92.32^{p} \pm 1.12$	
Vinblastine sulfate	0.5	$23.69^{a} \pm 1.38$	0.89	$24.76^{i} \pm 1.38$	1.14
	0.75	$38.18^{b} \pm 1.21$		$38.65^{k} \pm 1.23$	
	1	$64.02^{d} \pm 1.28$		$49.41^{n} \pm 1.32$	
	1.5	$78.34^{h} \pm 1.17$		$68.61^{r} \pm 1.41$	
	2	$91.65^{f} \pm 1.44$		$91.78^{p} \pm 1.57$	

Mean in the same column followed by the same letter are not significantly different at ($P<0.05$)

Additionally, the extract from the leaves showed significant selectivity towards the A-549 and HCT-116 cancer cell lines as opposed to MRC-5 cells, which are healthy normal cells. As seen in Table 5, to further characterize selectivity of plant extract, its effect was investigated on normal healthy MRC-5 cells (Abouzied *et al*., 2021). Aerial parts extract of EST was found to have lower cytotoxic activity against normal healthy MRC-5 cells, which indicate that EST extract possess low toxicity towards normal healthy cells that is a vital property for any cytotoxic drug. Both A549 and HepG-2 cells demonstrated high degree of selectivity, with EST extract being approximately two times more selective. It showed highest results with A549 cells. High selectivity suggests that EST extract from aerial parts is less damaging to healthy cells. Due to these promising outcomes against A549 cells, we looked into the mechanism of action of *Ephedra transitoria* extract on cancer cell lines.

Table 5. IC_{50} values of *E. transitoria* aerial parts extracts against MRC-5 normal cells

IC_{50} (µg/ml)[a]		Selectivity Index (SI)[b]			
	MRC-5	HepG-2	A-549	HCT-116	MCF-7
EST extract	45.22 ± 1.62	2.06	2.30	0.96	0.89

a IC_{50} values are reported as the mean ($IC_{50} \pm SD$) *of three experiments. B SI = (*IC_{50} of MRC5)/ (IC_{50} of cancer cell).

EST methanolic extract induced S phase cell-cycle arrest in A-549 cells

We chose to characterize the bioactivity of this extract by showcasing how it affects the cell cycle because aerial parts' extract exhibited the highest cytotoxic activity against A-549 cancer cell lines. In an effort to investigate the mechanism of plant extract as an anticancer agent, the levels of checkpoints were measured to clarify the efficacy of methanolic extract of plant in inducing a cell rest and entering apoptotic phase, IC_{50} of plant extract and lung cancer cell lines were examined. Figure 4 shows that A-549 cancer cells treated with an EST methanolic extract had a higher percentage of cells in the S-phase (48.31% as opposed to 36.17% in control cells). This increase in percentage of cells in percentage of cells in S-phase relative to the control indicates that EST extract induces S-phase cell cycle arrest in lung cancer cell lines. These findings were confirmed with the data obtained from Annexin-V FITC assay cell cycle analysis. Therefore, it can be deduced that EST extract induced S-phase arrest and apoptosis in A-549 cells, as shown in Figure 5.

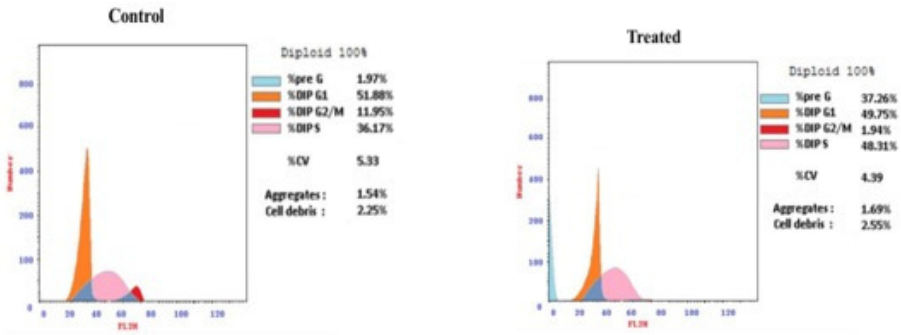

Figure 4. Cell-cycle histograms showing effect of EST methanolic extract on cell-cycle progression in A549 cancer cells after 72 h of treatment at IC_{50} concentration

EST methanolic extract activated caspase-dependent and Bax and p53 mediated apoptosis in A-549 cells

Cell cycle analysis demonstrated that EST methanolic extract caused apoptosis in A-549 cells. Therefore, to establish that extract induced apoptosis, an annexin V/propidium iodide (PI) apoptosis assay was carried out on A-549 cell lines (Figure 5). It was found that there were more necrotic cells (5.50% versus 1.24% in untreated cells). Therefore, we may conclude that the methanolic extract from aerial portions caused the majority of cancer cells to die via inducing apoptosis and just a small number of cells to die by necrosis.

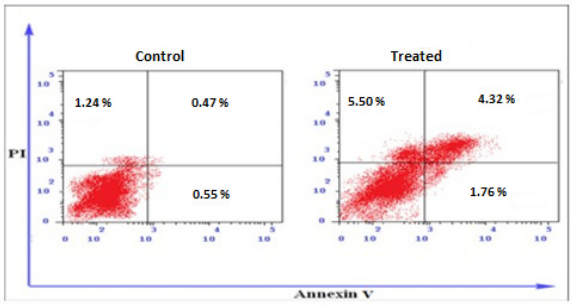

Figure 5. Apoptosis quadrant plots showing apoptotic effects of EST extract on A549 cancer cells upon treatment with extract at IC_{50} concentration for 72 h

Apoptosis-related proteins like caspase-3 and p53, were shown to be induced by EST methanolic extract. Activation of caspases is typically regarded as the primary indicator of apoptosis. Apoptosis is one of the many anti-proliferative mechanisms that are impacted by the p53 protein, which is responsible for tumor suppression. The inhibition of cancer depends on the activation of this protein (Khazaei *et al*., 2017). After treating A549 cancer cells with EST methanolic extract, the results of a western blot analysis revealed high levels of protein expression for cleaved caspase-3. This explains induction of apoptosis in treated A-549 cancer cells and correlates with Table 6 results from the Annexin V/PI experiment.

Table 6. Effect of EST methanolic extract on expression of apoptotic proteins in lung cancer cell lines

Samples	Protein expression (normalized to β-actin)*			
	Bax	Bcl2	Caspase-3	P53
Control (A-549 cell lines-untreated)	3.29 ± 0.32	8.19 ± 0.45	49.41± 3.22	4.31 ± 0.14
EST Extract	14.42± 0.43	3.92± 0.37	132.94 ± 4.13	13.19 ± 1.56

***** Average of 3 determinations by ELISA technique

The anticancer efficaciousness of EST methanolic extract was evaluated down to the level of morphological structure and cell cycle checkpoints. The expression of apoptosis-related proteins, such as pro-apoptosis protein Bax, anti-apoptosis protein Bcl-2, tumour suppressor p53, and caspase-3, as caspase activation plays a significant role in cell death, as well as the level of biochemical characteristics of apoptotic cells, which are regulated by several apoptotic factors (Ramadan *et al*., 2019; Pisani *et al*., 2020). The results indicated that when EST aerial parts extract was applied, Bax was up-regulated and Bcl2 was down-regulated in comparison to control. Moreover, EST extract remarkably upregulated expression level of cleaved caspase-3 when compared to control cells Multiple stress signals are integrated into a variety of distinct anti-proliferative responses by the p53 tumor suppressor. Activating apoptosis is one of p53's most crucial roles, and interfering with this mechanism can accelerate the growth of tumors (Pisani *et al*., 2020). The results in Table 6 show that when our chosen EST extract was added to the control, the p53 level virtually increased by 3 folds.

Conclusions

Based on the current study's findings, some bioactive phytochemicals from ephedra transitoria aerial parts crude extracts have the potential to function as antioxidants, antibacterial, as well as anticancer. The results show that, methanolic extract of ephedra transitoria exhibited good antioxidant, antibacterial and anticancer activity when compared to ascorbic acid, gentamycin and vinblastine sulfate standards. Good cytotoxic activity was observed against both HepG2 and A549 cancer cells. Additionally, the BCl2 gene is downregulated in the A549 cell line while BAX genes are regulated, producing apoptosis. This study also indicated that overexpression of the BAX gene and decreased expression of the BCl2 gene inhibited the proliferation of A549 lung cancer cells. Moreover, apoptosis results showed that plant extract produced S-phase arrest and death in A549 cells, which may be attributed to presence of some bioactive phytochemicals that were separated and identified by GC-MS analysis technique. It will take more research to separate pure beneficial chemicals from this crude extract, as doing so could potentially result in a brand-new lung cancer treatment.

Authors' Contributions

Conceptualization: KM and FA; Methodology: KM and AM; Formal analysis: KM and FA, Investigation: AM and LA; review: SM and AS: Writing – original draft. KM and MK; review & editing: TA and GA.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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