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Antiviral, Antifungal, and Antibacterial Potential Activities of *Ephedra Sinica* in *Vitro*

Mohamed M. Deabes[1], Abdou K. Allayeh[2*], Mohamed M Seif [1], Abdel-Hamied M. Rasmey[3] and Khayria M. Naguib[1]

[1] *Food Toxicology and Contamination Department,* [2] *Virology lab 176, Water Pollution Research Department, National Research Centre, El Buhouth St, Dokki, 12622-Cairo,* [3] *Botany and Microbiology Department, Faculty of Science, Suez University, Suez, Egypt.*

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Abstract

This study was conducted to assess the antiviral, antifungal and antibacterial potential of extracts from *Ephedra sinica*. Plant extract activity was screened for four bacterial, three fungal and one viral pathogen including: *Klebsiella pneumoniae, Enterobacter cloacae, Serrattia marcescens, Escherichia coli, Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger and* Coxsackie B_3 virus. The findings disclosed promising inhibitory impacts against all pathogens. Ethanol-water extract showed that the greatest inhibition zones (mm) against bacterial and fungal species ranged from 20.7 to 25.0 and 29.0 to 35.0 at a concentration of 150 µl/ml respectively, similar to methanol-water extract inhibition zones against the same pathogens. Interesting outcomes against Coxsackie B_3 virus was observed. For ethanol-water extract (51.6%), followed by ethanol extract (46.6%) and methanol extract (41%) during zero-time infection, the greatest viral inhibition activity was documented. During virucidal process, a limited action of inhibition was observed for ethanol extract (39.4%), methanol extract (36.8%), and ethanol-water extract (31%). No viral inhibition action was notarized in both pre- and post- infection processes. The findings of this study indicated that *Ephedra sinica* is an excellent source of bioactive substances against a wide range of various microbial pathogens.

Keywords: *Ephedra sinica*, Antiviral, Antibacterial, Antifungal

1. Introduction

The quest for new substances with high efficacy, low toxicity, and minor side effects must proceed against microbial diseases due to the global need for these substances to solve drug resistance issues. Growing interest in natural products derived antimicrobial agents still remains the best resource for future advancement as powerful and safe agents for modern medication (Nagai *et al*., 2011; Allayeh *et al*., 2015 and 2018). Particularly the medicinal plants; *Ephedra sinica* is regarded one of the most important plants in traditional medicine. *Ephedra sinica* has been used in several disease treatments for over 5000 years (Naidu, 2000; Soni *et al*., 2004). The dried plant has traditionally been used as a tea to treat asthma, cough, fever, urinary incontinence, lack of sweating, and edema reduction. Furthermore, the *Ephedra* plant includes bronchial dilator, ephedrine, and high quantities of proanthocyanidins with prominent biological activities such as alkaloids, such as bronchodilation and vasorelaxation. Proanthocyanidins were used as antimicrobial, antioxidant, anti-inflammatory, immunosuppressive, antiviral, anti-invasive, antiangiogenic, antitumor, and cytotoxic activity, especially against cancer cell lines such as SGC-7901, HepG2, and HeLa. *Ephedra sinica* has recently been used in the United States market as a supplement for weight loss and energy gain (Hyuga *et al*., 2004 and 2007; Zhang *et al*., 2018). Several reports have documented the antimicrobial activities of *Ephedra* genus against various microorganisms species, including Bacterial species such as; *Staphylococcus aureus, Bacillus anthracis, B. diphtheriae, B. dysenteriae, B. typhosus, Pseudomonas aeruginosa, Lactobacillus acidophilus*, and *Lactobacillus casei*; Fungal species such as *Aspergillus parasiticus, Saccharomyces cerevisiae, Candida albicans, Candida utilis*; viral species such as avian influenza virus (Bagheri-Govkosh *et al*., 2009; Soltan and Zaki, 2009; Lee *et al.*, 2009; Fazeli-Nasab and Mousavi, 2019). This work will explore the prospective antibacterial, antifungal and antiviral activities of various *Ephedra sinica* crud extracts against eight species of various pathogens, including *Klebsiella pneumoniae, Enterobacter cloacae, Serrattia marcescens, Escherichia coli, Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger,* and Coxsackie B_3 virus. We used this virus as model to study the prospective antiviral activity due to its association with several human diseases such as deadly viral myocarditis, hepatitis, meningitis, gastroenteritis, hand foot mouth disease and fibrosis cardiomyopathy that could advance toward heart failure with no effective medication so far.

* Corresponding author e-mail: drallayeh@yahoo.com.

2. Materials and Methods

2.1. Plant Extraction Preparation

Ten grams of *Ephedra sinica* (stem part) powder were extracted by 100 ml of each (ethanol (ET), ethanol: water (ET: H_2O), methanol (MeoH) and methanol: water (MeoH: H_2O)) at room temperature for 24 hours in a shaker. Finally, the extract was concentrated under vacuum with a rotary evaporator and sterilized by using a 0.22 µm syringe filter.

2.2. Antimicrobial Activity Preparation

The experimental bacteria isolates including, *Klebsiella pneumoniae, Enterobacter cloacae, Serrattia marcescens, and Escherichia coli* were kindly provided by the Department of Botany and Microbiology, College of Science, Suez University, Egypt. The microbial strains were maintained on nutrient agar slant at 4°C until antimicrobial testing was conducted. According to Sethiet *et al.*, (2013), the antibacterial activity was determined by agar well diffusion assay as the following; Nutrient agar medium was poured at 45°C in pre-seeded sterilized plates with 500 µl of 18 h culture of each bacterial species. Poured plates seeded with test organisms were rotated to allow homogenous spread of the inoculum and left for 30 min at room temperature to solidify. Using a cork-borer, wells of 5 mm in diameter were made in the medium of these plates and injected by *Ephedra* extracts (50, 100 and 150 µl) for each concentration in these wells under aseptic conditions. The Petri-dishes were held in a refrigerator for two hours to allow antimicrobial agent to spread homogenously before the organism was allowed to grow (Selim *et al*., 2011). The plates were incubated at 37°C overnight. A positive test showed the appearance of inhibition zone and the diameters of the inhibition zone around the well were measured.

2.3. Antifungal Activity Preparation

Ephedra extracts' antifungal activity was tested against *Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger* using the agar well diffusion method. Briefly, fungi spore suspensions were prepared and adjusted approximately to 10^6spore/ml. Potato dextrose agar (PDA) (Sigma-Aldrich, USA) plates were inoculated with 1 ml of designated fungal spore suspension. Wells of 5 mm diameter were produced on the PDA surface and filled with *Ephedra* extracts at concentration 50, 100 and 150 µl and incubated at 28°C for 72 hours. Fungal growth control was prepared using PDA medium inoculated with spore suspension without any extracts. After incubation, the plates were tested for the inhibitory zones (mm) of the mycelial growth around the wells (Kavanagh, 1972). All experiments were performed in triplicates.

The PDA medium was prepared with distinct plant extract concentrations of 50, 100 and 150 µl by adding extract to the melted medium, followed by the addition of Tween 80 to disperse the extract into the medium. Thirty milliliters of the medium were poured into glass Petri-dishes (9 x 1.5 cm). Each Petri-dish was inoculated at the center with a mycelial disc (6 mm diameter) of the fungal culture. Positive control plates were inoculated following the same procedure. Plates were incubated at 28°C for seven days and the colony diameter was observed daily.

2.4. Antiviral Activity Preparation

Confluent monolayer vero cell (Holding Company for Biological Products and Vaccines, Egypt) was used for the propagation of Coxsackie B_3 virus which was kindly provided by Dr. Mohamed Shaheen, (Shubhada Bopegamage's Lab, Slovak Medical University, Slovakia). This cell line cultured in Dulbecco's modification of eagle's medium DMEM, 13.48 g of DMEM powder was dissolved in one liter of de-ionized H_2O. This culture medium was supplemented with 0.1% L-glutamine, heat-inactivated fetal bovine serum (FBS) from Gibco-BRL in 10% or 2% to prepare growth or maintenance medium, respectively. The following antimicrobials were added: Penicillin/Streptomycin at the final concentration of 50 µg/ml, and Fungal-zone at the final concentration of 2.5 µg/ml obtained from the Gentech Company in Egypt. Using a 0.22 µm syringe filter, fully medium was filtered and sterilized. Virus titer was determined as the next by using Endpoint (limiting) dilution method. At room temperature, a tube of viral stock suspension was thawed. Once thawed, 100 µl of the stock viral solution was serially diluted into a medium sequence of tenfold dilution. Confluent cells (10^4 cells/ well) prepared in 24-well plates were inoculated with 100 µl of each dilution in triplicate. The plates were then incubated at 37°C (5% CO_2) and observed daily for the development of characteristic CPE. The lowest dilution of viral stock suspension that could still cause 50% of a CPE was taken as the maximum dilution that would result in infection. This was called the $TCID_{50}$ because it was the minimum concentration of viral particles needed to detect viral CPE and provide a rough indication of the overall concentration of virus present within the stock suspension. The endpoint dilution, expressed as $TCID_{50}$/ml, was calculated by using Reed-Muench formula (Reed and Muench, 1938; Flint *et al*., 2003).

2.4.1. Cytotoxicity and Cell Viability

Adding various dilutions of tested extracts to the media can trigger cell modifications. Thus, to ensure that all morphological changes are reported, the maximum concentration of tested extracts at which the cells will display no morphological changes will be determined. Cell suspensions were seeded in 24-well plates and left at 37°C for 24 hours in 5% CO_2 incubator. Upon completion of the confluence, distinct dilutions of the tested extracts were added. The plates then incubated at 37°C in 5% CO_2 atmosphere for two days and cell morphology changes were observed daily. Using the CPE scoring scheme, evidence of morphological change (such as loss of monolayer, granulation, and vacuolization in cytoplasm) was reported (Vijayan *et al*., 2004). All assays were conducted with approximately $3x10^4$ cells per well in triplicates in 24-well plates.

2.4.2. Cytopathic Inhibition Assay

Vero confluent 24-well plats have been infected at 37°C for 90 min with 100 µl of stock Coxsackie B_3 virus. Then, 100 µl of the extracts were added. For each dilution, three wells were used and 100 µl of the maintenance medium were added per well. Ultimately, plats were incubated for three days until full CPE was observed. The inhibition of CPE was determined by the antiviral activity relative to control and expressed by the formula of Reed

and Munech, (1938). The mechanism of antiviral activity against Coxsackie B_3 virus was investigated in triplicates using cytopathic inhibition assay with some modifications during each process as the next;

2.4.2.1. Pre-Viral Infection Process

Vero cells were cultivated in 24-well plates and inoculated in 5% CO_2 incubator with 100 µl of tested extracts for 90 min at 37°C. The medium was aspired and 100 µl of Coxsackie virus was inoculated for 90 min at 37°C in 5% CO_2 incubator. The maintenance medium was added and further incubation time for 3 days at 37°C in 5% CO_2 incubator. Antiviral activity was determined by the inhibition of CPE related to the control according to Hai-Rog Xiang *et al.*, (2012) and expressed by the formula of Reed and Munech, (1938).

2.4.2.2. Zero-Time Viral Infection Process

One hundred micro-liter of Coxsackie B_3 virus was inoculated into the confluent monolayer of vero cells accompanied by the same volume of tested extracts at 37°C in 5% CO_2 incubator for 3 days until CPE was observed. Antiviral activity was determined by the inhibition of CPE liked to control and expressed by the formula of Reed and Munech as outlined in the prior section.

2.4.2.3. Post-Viral Infection Process

This test was performed with the following variations as mentioned in the prior sections: Confluent of Vero cell line was cultivated in 24-well plates and handled for 90 min with 100 µl of Coxsackie B_3 virus in 5% CO_2 incubator at 37°C. With 100 µl of tested extracts, cells were washed and overlaid. Until CPE was observed, the plates were incubated for 3 days.

2.4.2.4. Virucidal infection process

The direct effect of extracts on Coxsackie B_3 virus was recognized by blending equal volumes of virus suspension with each extract and incubated at 37°C in 5% CO_2 atmosphere for 90 min prior cell infection. The mixture has been introduced to the cells. The same processes in the previous sections have been performed.

2.5. GC/MS Analysis

The assessment was conducted using a GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly-siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thickness). The carrier gas was helium with the linear velocity of 1ml/min. The temperature of the injector and detector was 200º C and 250º C, respectively. The volume injected size is 1µl. The operating parameters for MS were as follows: ionization potential 70 eV, interface temperature 250º C, and acquisition mass range 50–800. Component identification was based on a comparison of their mass spectrum and retention time with those of the authentic compounds and by computer matching with NIST and WILEY libraries as well as comparison of the fragmentation pattern of the mass spectral information with those reported in the literature (Sanatana *et al*., 2013).

2.6. HPLC Conditions

The polyphenols determination from the four *Ephedra* extracts was performed using an Agilent 1260 series. Column C18 (4.6 mm x 250 mm i.d., 5 µm) was used to separate. The mobile phase consisted of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A); 0–5 min (80% A); 5-8 min (40% A); 8-12 min (50% A); 12-14 min (80% A) and 14-16 min (80% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 35 °C (Amakura *et al*., 2013).

2.7. Statistical analysis

All experiments were performed in triplicate and the data expressed as means of ± standard deviation (SD) using Microsoft Excel software V2007.

3. Results and Discussion

The genus of *Ephedra* is widespread globally and its composition depends mainly on its species, harvest dates, fractionation processes, and geographical area. In this manner, their pharmacological effect could be quite-species specific. Most of the previous *Edephra* reports have been performed on *Ephedra sinica*, the pharmacological characteristics of which seem to come basically from significant alkaloids, ephedrine, and pseudoephedrine (Walaa *et al*., 2019). This study is one of very restricted work to our understanding that the antimicrobial actions of *Ephedra sinica* explored. Based on the results of the current study, all *Ephedra sinica* extracts have been shown to inhibit wide range of pathogens as the next in the following sections.

3.1. Antibacterial Activity

To explore *Ephedra sinica*'s crud extracts for antibacterial activity; all crud extracts showed prominent inhibition zones against all bacterial species. Indeed, the largest inhibition zones (mm); 25.0, 22.3, 20.7, and 21.0 have been reported for ethanol: H_2O fraction at 150 µg/ml concentration against *Serrattia marcescens*, *Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*, respectively. Although at the same concentration, methanol: H_2O fraction also displayed elevated inhibition zones; 24.3, 21.0, 19.6, and 20.0 against the same bacterial species. In comparison to prior extracts, inhibition zones of absolute ethanol extract (18.3, 17.0, 15.7 and 14.6) accompanied by absolute methanol extract (21.6, 20.0, 18.0 and 18.6) showed a moderate inhibition of the same species (Table 1). In this respect, Plant *Ephedra sinica* could be regarded as an excellent growth inhibitor against bacterial species. On the other hand, ethanol: aqueous extract has been discovered to be more effective against bacterial species compared to methanol: aqueous extract that also works against the same bacterial species. This outcome is in complete agreement with the previous report about ethanol and aqueous extractions of *Ephedra gerardiana* against *B. atrophaeus, Escherichia coli, Staphylococcus aureus* and *K. pneumonia* (Khan *et al*., 2017). Unlike *Ephedra gerardiana's* ethanol extract against *E. coli, S. aureus*, and *P. aeruginosa* also exhibited prominent inhibition zones (Kumar and Sigh, 2011).

Table 1: Inhibition zones (mm) formed by E.S extracts against Bacterial spp.

Extract	Concentration (µl)	Inhibition zone (mm)			
		Serratia marcescens	*Escherichia coli*	*Enterobacter cloacae*	*Klebsiella pneumoniae*
MeOH	50	10.7±1.7	8.6±1.5	8.5±1.1	11.0±2.0
MeOH:H_2O		12.3±2.1	10.6±1.2	10.6±2.08	13.0±1.9
ET		8.0±1.8	7.3±2.1	7.0±1.5	8.0±1.0
ET:H_2O		13.0±1.8	12.3±2.3	10.0±1.5	11.5±1.5
MeOH	100	16.0±1.5	13.0±1.52	10.0±1.1	12.0±1.0
MeOH:H_2O		19.0±2.6	16.0±1.0	14.7±1.52	14.6±2.5
ET		13.0±2.1	10.6±1.6	8.7±1.5	10.7±1.3
ET:H_2O		19.6±2.1	16.7±1.6	15.7±2.08	15.0±2.0
MeOH	150	21.6±1.5	20.0±1.7	18.0±1.2	18.6±1.7
MeOH:H_2O		24.3±1.15	21.0±2.0	19.6±1.8	20.0±1.5
ET		18.3±2.0	17.0±2.0	15.7±2.3	14.6±1.5
ET:H_2O		25.0±2.0	22.3±2.5	20.7±2.1	21.0±2.0

3.2. Antifungal Activity

All crud extracts have been assessed for antifungal activity against *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger* based on powerful prospective antibacterial activities. Hence, significant antifungal activity or elevated inhibition zones were documented in this study. Inhibition zones of 29.0, 35.3, and 32.1 (mm) for Ethanol: H_2O fraction (150 µg/ml) were disclosed against *Aspergillus flavus*, *Aspergillus ochraceus*, and *Aspergillus niger*, followed by inhibition zones (26.5, 32.5 and 28.5) of methanol: H_2O fraction, respectively, whereas inhibition zones of absolute methanol (22.6, 29.5 and 25.0) followed by absolute ethanol inhibition zones (18.5, 23.0 and 15.0) showed average inhibition activity against the same fungal species compared to prior extracts at different concentrations (Table 2). Since the growth of the *Aspergillus* species at different levels of 50,100 and 150 µg / ml was considerably inhibited, the current work suggests that *Ephedra sinica* could be used as a therapy against *Aspergillus* species. Our findings are comparable to the previous report on the essential oils of *Ephedra* major which exhibited significant inhibition of fungal growth and Aflatoxin production according to Razzaghi-Abyaneh *et al*., (2009). For essential oils of *Ephedra* major at the concentration of 1000 µg/ml, powerful inhibition of *Aspergillus parasiticus* and aflatoxin production was reported in other studies (Bagheri-Govkosh *et al*., 2009; Abou El-Soud *et al*., 2015; Deabes *et al*., 2018). On the other hand, few studies showed that there was no obvious inhibition impact of the *Ephedra breana* fractions against *Aspergillus flavus* at 1000 µg / ml levels (Feresin *et al*., 2001). Another study showed that *Ephedra procera* methanol extract could not influence *Aspergillus flavus* growth at concentrations of 1000 µg/ml (Fazly-Bazzaz and Haririzadeh, 2003). This may refer to the variations in the *Ephedra* plant's compositions or species.

Table 2: Inhibition zones (mm) formed by E.S extracts against Fungi spp.

Extract	Concentration (µl)	Inhibition zone (mm)		
		A. flavus	*A. ochracues*	*A. niger*
MeOH	50	9.0±1.8	15.0±2.1	11.5±1.5
MeOH:H_2O		14.7±2.2	18.5±1.1	16.0±1.07
ET		6.5±1.5	9.3±1.5	7.0±1.0
ET:H_2O		14.5±1.8	19.0±1.5	16.5±1.5
MeOH	100	16.5±1.5	23.7±1.0	18.1±1.3
MeOH:H_2O		21.0±2.0	27.0±1.5	24.5±1.5
ET		9.5±1.0	15.0±1.7	11.5±1.0
ET:H_2O		23.5±2.1	28.6±1.4	27.1±1.5
MeOH	150	22.6±1.0	29.5±1.1	25.0±1.5
MeOH:H_2O		26.5±1.5	32.5±1.0	28.5±1.0
ET		18.5±1.0	23.0±1.9	15.0±2.0
ET:H_2O		29.0±1.0	35.3±2.0	32.1±1.5

3.3. Antiviral Activity

No cytotoxic effect was observed for all the extracts using Vero cell lines. Contrary to the outcomes of antibacterial- or antifungal- experiments in the present study, interesting phenomena was reported during antiviral experimentation; during pre-infection or post-infection mechanisms, neither crude extract nor any fraction showed significant antiviral activity against Coxsackie B_3 virus. But during zero-time infection and virucidal processes for particular extracts, an inhibition activity was reported as shown in (Table 3). The greatest inhibition activity was documented for ethanol: H_2O (51.6%) followed by absolute ethanol (46.6%), absolute methanol (41%), respectively during the zero-time process. For absolute ethanol, absolute methanol, and ethanol: H_2O fractions, 39.4%, 36.8%, and 31% of inhibition activity were reported during the process of virucidal infection, respectively.

Table 3: Antiviral activity of E.S extracts against Coxsackie B_3 virus.

Extract	% of CPE Inhibition of Coxsackie B_3 virus			
	Pre-infection	Post-infection	Virucidal infection	Zero-time infection
Methanol extract	15.2%	11%	36.8%	41%
Methanol : H_2O	5%	2%	31%	33.5%
Ethanol extract	13.8%	16.4%	39.4%	46.6%
Ethanol : H_2O	5.5%	9.3%	31%	51.6%

The crud extracts of *Ephedra sinica* have moderate antiviral activity against Coxsackie B_3 virus in *vitro* compared to the outcomes of both antibacterial and antifungal tests in the current work. Contrary to the previous study conducted on plant *Ephedra aphylla* collected from Egypt, mild proportion of antiviral activity (40.7%) against Herpes virus type I was also documented (Khaled *et al*., 2018). According to Mantani *et al*., (1999), the inhibition may due to the inhibitory effect of the *Ephedra* on the acidification of intracellular elements such as lysosomes that have capability to inhibit the growth of influenza virus. In another study, (Sumiko *et al*., 2016) confirmed that the ephedrine alkaloids-free *Ephedra* Herb extract has significant antiviral activity against influenza virus and suggested obtaining a license approval for the therapeutic use of ephedrine alkaloids-free *Ephedra*.

3.4. GC/MS And HPLC Analysis

According to GC/MS analyses, the retention indices (RI) and the composition % of twelve components were identified in *Ephedra sinica* out of which {Lupeol & 2-Nonaprenyl-6-methoxyphenol & Glycodeoxycholic acid & Ledene} and {5, 7-dimethoxyflavone & 4-(Ethylamino)-6-(methylsulfanyl)-1, 3, 5-triazin-2-ol} were the principle principal? Components found in ethanol-water and absolute ethanol extracts, respectively. The profile of GC/MS for methanol-water and absolute methanol extracts was completely different from the previous extracts. Here, twenty-one and eleven components were documented for methanol-water and absolute methanol extracts, respectively (Table 4). On the other hand, higher polyphenol (Gallic acid, Coffeic acid, Syringic acid) content was observed in aqueous-organic solvents extracts in contrast to their absolute solvents by using HPLC (Table 5). Extract of Ethanol-water had the largest polyphenol content followed by extract of methanol-water, while there were no several peaks in both absolute extracts of ethanol and methanol (Figures 1-5). These are comparable to the results of the previous report conducted on black tea and which showed that 50 % of aqueous organic solvents have extracted more polyphenolic compounds than absolute organic solvents (Michael *et al*., 2016). This phenomenon may be because water swells the plant material and increases extractability by allowing the solvent to penetrate more easily into the solid matrix (Horax *et al*., 2010). To our knowledge, the phenolic compounds may interact with the membrane proteins, which changes membrane permeability, and causes cell death or penetrates the pathogen itself and coagulates its content according to Tian *et al*., (2009). These mechanisms support our outcomes and explain why the aqueous-ethanol extracts have significant antibacterial, antifungal and antiviral activities in this work more than absolute solvent, which may be due to a high content of polyphenol.

Table 4: GC/ MS Profile for each E.S extract according to Retention time and constituents concentration (%)

A- Absolute Ethanol Extract (ET)

No.	Name of compounds	Molecular Formula	Retention time	Ratio (%)
1	Thiourea,N-(4-butoxyphenyl)-N'-[4-(dimethylamino)phenyl]	$C_{19}H_{25}N_3OS$	6.008	1.05
2	1'-Hydroxymidazolam	$C_{18}H_{13}ClFN_3O$	14.421	1.31
3	Tetrahydro-L-biopterin	$C_9H_{15}N_5O_3$	14.567	1.3
4	4',6-Dimethoxyisoflavone-7-O-β-D-glucopyranoside		14.673	1.67
5	Cholesta-5,7-dien-3-ol, (3β)-	$C_{27}H_{44}O$	15.146	6.2
6	Noscapine	$C_{22}H_{23}NO_7$	15.594	1.23
7	Methyl farnesoate	$C_{16}H_{26}O_2$	19.147	3.01
8	Squalene	$C_{30}H_{50}$	19.786	1.0
9	5,7-dimethoxyflavone	$C_{17}H_{14}O_4$	20.565	25.89725
10	Trp-Cys-Arg, Tryptohan , cystein, arginine		21.437	3.5
11	4-(Ethylamino)-6-(methylsulfanyl)-1,3,5-triazin-2-ol	$C_6H_{10}N_4OS$	22.194	24.51656
12	3-Methoxy-6,7,8,9-tetrahydrodibenzo[b,d]furan-2-ol	$C_{13}H_{14}O_3$	22.842	8.716873

B- Ethanol-Water Extract (ET: H2O)

No.	Name of compounds	Molecular Formula	Retention time	Ratio (%)
1	Salicylic acid	$C_7H_6O_3$	3.445	9.04
2	Pyrocatechol	C_6H_4-1,2-$(OH)_2$ $C_6H_6O_2$	3.539	5.13
3	Hydroquinone	$C_6H_6O_2$ C_6H_4-1,4-$(OH)_2$	6.308	6.72
4	4-Pyridinecarboxaldehyde	C_6H_5NO	3.755	4.37
5	7,3',4',5'-Tetramethoxyflavanone	C18H16O8	6.081	1.36
6	Quercetin 3',4',7-trimethyl ether	C18H16O7	13.199	1.47
7	Propyl gallate	$C_{10}H_{12}O_5$	14.462	2.63
8	2-Nonaprenyl-6-methoxyphenol	$C_{17}H_{24}O_2$	16.522	14.13
9	Glycodeoxycholic acid	$C_{26}H_{43}NO_5$	1.428	11.43
10	Ledene	$C_{15}H_{24}$	21.327	11.12
11	Lupeol	$C_{30}H_{50}O$	21.954	19.75
12	Betulin	$C_{30}H_{50}O_2$	23.091	3.62

C- Absolute Methanol Extract (MeoH)

No.	Name of compounds	Molecular Formula	Retention time	Ratio (%)
1	Pyrocatechol	C_6H_4-1,2-$(OH)_2$ $C_6H_6O_2$	4.02	14.47
2	DL-Homocysteine, S-ethyl-	$C_6H_{13}NO_2S$	4.207	7.59
3	1-Aminothiourea	CH_5N_3S	4.529	1.899823
4	Benzoyl isothiocyanate	C_8H_7NS	4.696	2.226332
5	Salsoline (Alkaloid)	C11H15NO2	5.804	1.058791
6	7,8-Dihydroneopterin	$C_9H_{13}N_5O_4$	13.936	1.201704
7	2-Acetyl-5-(tetrahydroxybutyl)imidazole	$C_9H_{14}N_2O_5$	14.103	2.353973
8	4',6-Dimethoxyisoflavone-7-O-β-D glucopyranoside		14.861	1.643087
9	Olomoucine	C_{15}H18N6O	16.136	1.17494
10	2,6-dimethyl-N-(2-methyl-α-phenylbenzyl)aniline	$C_{10}H_{15}N$	20.349	44.02967
11	Betulin	$C_{30}H_{50}O_2$	22.529	1.086693

D- Methanol-Water Extract (MeoH: H2O)

No.	Name of compounds	Molecular Formula	Retention time	Ratio (%)
1	Epinephrine	$C_9H_{13}NO_3$	3.906	3.164819
2	Vitexin	$C_{21}H_{20}O_{10}$	3.979	3.9
3	Nopol (terpene)	$C_{11}H_{18}O$	4.256	9.81479
4	Benzo[h]quinoline, 2,4-dimethyl-	$C_{15}H_{13}N$	5.393	6.24
5	7,3',4',5'-Tetramethoxyflavanone	$C_{19}H_{20}O_6$	6.326	4.602642
6	8-Carboxy-3-methylflavone	$C_{17}H_7D5O_4$	6.99	5.2
7	4'-Hydroxychalcone	$C_{15}H_{12}O_2$	7.739	6.72
8	3-(2-Benzothiazolyl)-6-methoxycoumarin	$C_{17}H_{11}NO_3S$	8.012	3.177727
9	Norepinephrine	$C_8H_{11}NO_3$	8.53	2.45
10	Vanillic acid	$C_8H_8O_4$	9.059	3.82
11	Phloroglucinol	$C_6H_6O_3$	10.119	3.140511
12	Quercetagetin-7-O-glucoside	$C_{21}H_{20}O_{13}$	11.968	3.607574
13	Cholic acid	$C_{24}H_{40}O_5$	12.877	1.80761
14	Tetrahydro-L-biopterin	$C_9H_{15}N_5O_3$	13.797	2.809424
15	5,7,3',4',5'-Pentahydroxyflavone	$C_{15}H_{14}$	14.429	3.711465
16	Gardenin	$C_{21}H_{22}O_9$	14.889	2.320525
17	7-Diethylamino-3-(3,4-dimethoxyphenyl) coumarin	C21H23NO4	15.37	2.170816
18	Quinine	$C_{20}H_{24}N_2O_2$	15.566	2.696559
19	5β,7βH,10α-Eudesm-11-en-1α-ol	$C_{15}H_{26}O$	15.59	3.245133
20	Baicalein trimethyl ether		15.806	4.48629
21	3,4-Dihydrocoumarin	$C_9H_8O_2$	20.393	1.769145

Table 5: HPLC Separation for Polyphenol content in E.S extracts.

Poly phenol	Concentration (µg/ml)			
	Methanol Extract	Methanol-Water Extract	Ethanol Extract	Ethanol-Water Extract
Gallic Acid	5.66	17.70	1.41	49.54
Catechin	0.0	0.0	0.0	3.27
Coffeic Acid	4.02	8.20	0.0	8.33
Syringic Acid	0.0	2.61	0.0	2.78
Coumaric Acid	0.90	1.41	0.0	1.45
Vanillin	1.9	1.77	0.0	1.79
Cinnamic Acid	0.52	0.45	0.19	1.11

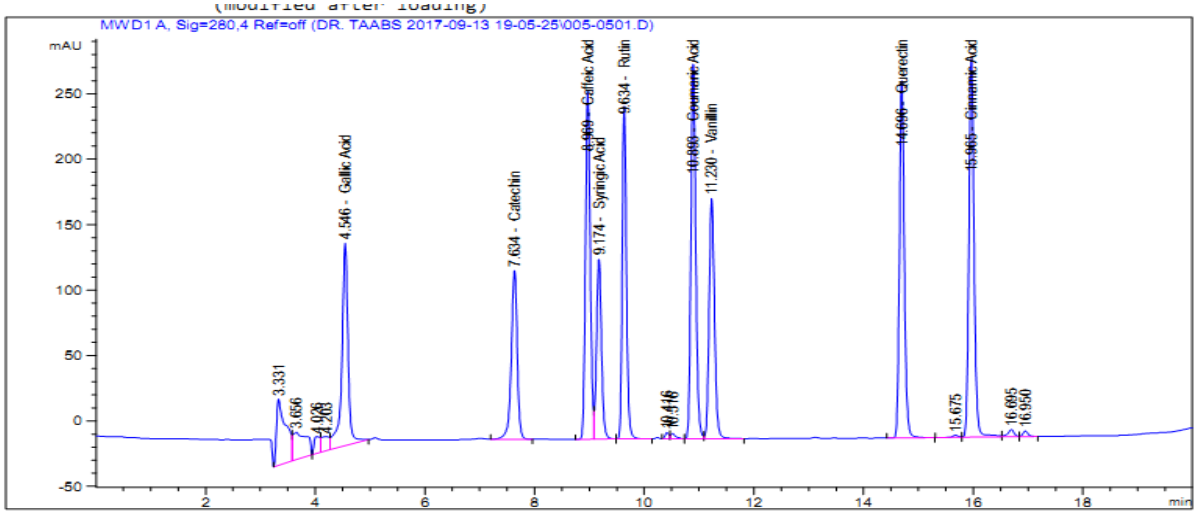

Figure 1: HPLC Profile for Polyphenol Standards

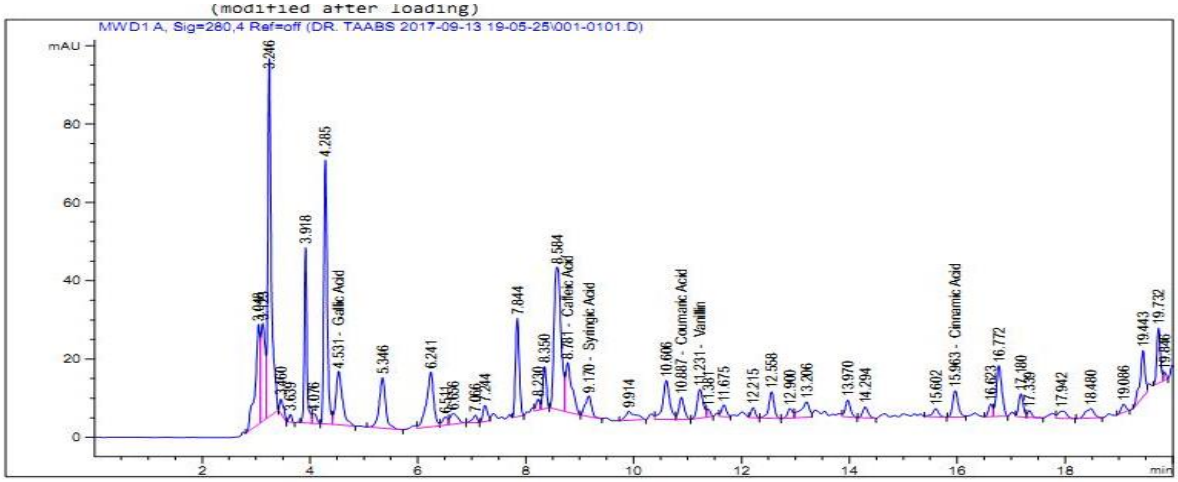

Figure 2: HPLC Profile for E.S extract by absolute Methanol Solvent

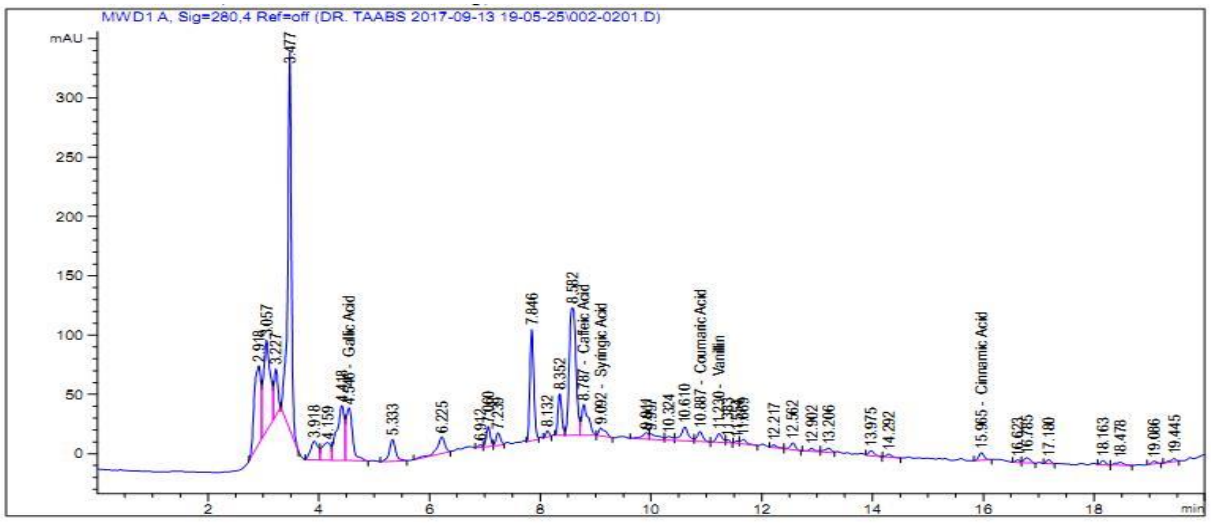

Figure 3: HPLC Profile of E.S extract by Methanol: water extract

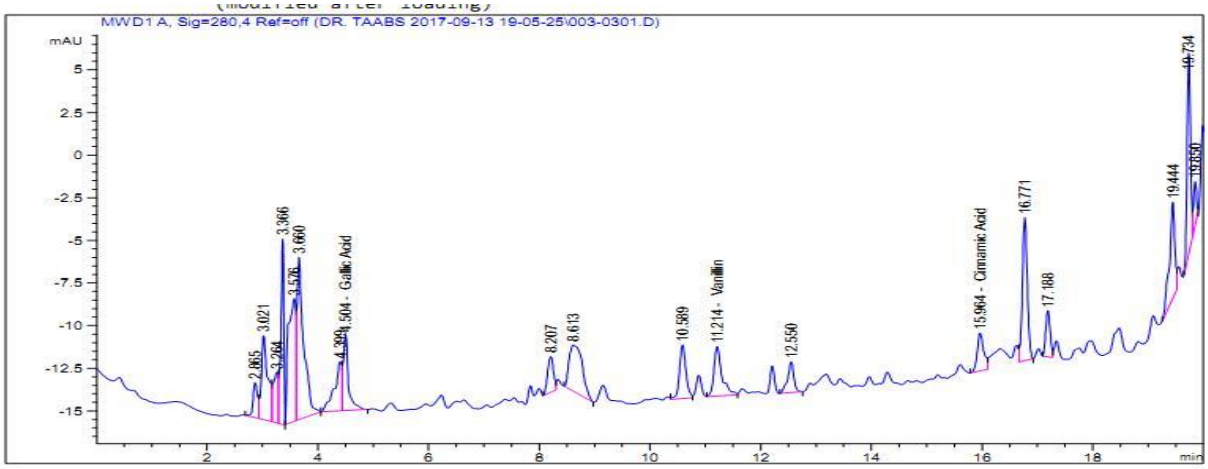

Figure 4: HPLC Profile of E.S extract by absolute Ethanol solvent

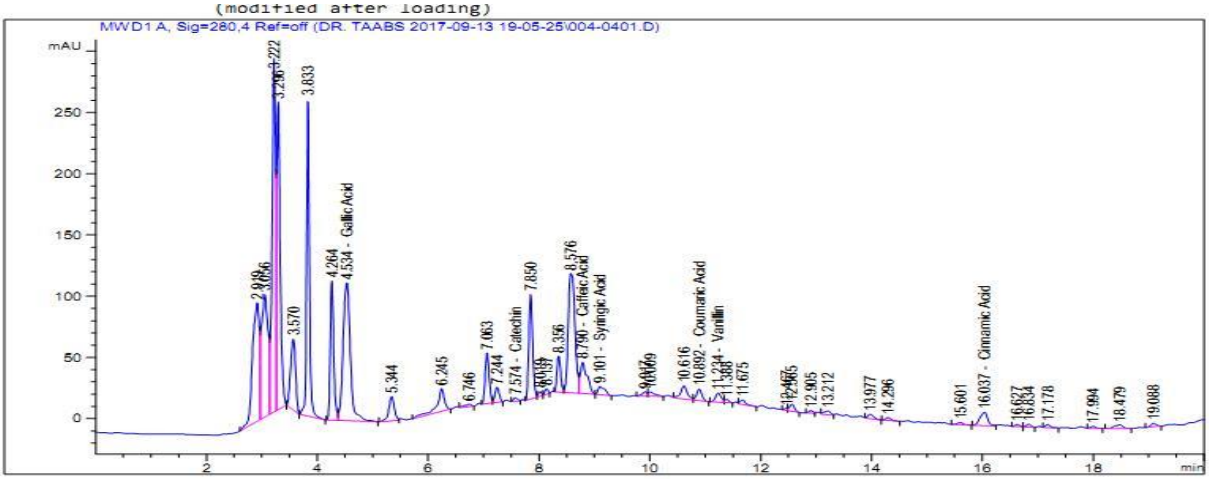

Figure 5: HPLC Profile of E.S by Ethanol: water extract

4. Conclusion

This study explored the aqueous-organic solvents and absolute solvent extracts from *Ephedra sinica* as well as antibacterial, antifungal and antiviral activities of these extracts. Our findings have revealed a significant inhibitory impact on all pathogen species. Ethanol-water extract showed that the greatest inhibition zones (mm) against bacterial and fungal species ranged from 20.7 to 25.0 and 29.0 to 35.0 at a concentration of 150µl/ml, respectively. For antiviral activity, 51.6% was the greatest inhibition documented for ethanol-water extract against coxsackie B_3 virus during zero-time process. We, therefore, suggest that aqueous-ethanol solvent can be used as a useful source for natural polyphenol and alkaloids with anti-wide range of pathogen species. Furthermore, mutagenic and carcinogenic investigations are necessary for safe human use.

5. Conflict of interest

The authors have declared that no competing interests exist.

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