COMPARISON OF TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT, ANTIOXIDANT ACTIVITY, ANTIMICROBIAL ACTIVITY AND CONTENT OF EPHEDRINE AND PSEUDOEPHEDRINE BETWEEN WILD *EPHEDRA ALATA* AND *EPHEDRA FRAGILIS* STEMS AND FRUITS IN PALESTINE

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Abstract

The genus Ephedra belongs to *ephedraceae* family that contains about 67 species, most of which are being used in traditional medicines. In this research, two species were analyzed: stems and fruits of *E. fragilis* and stems of *E. alata*. Stems of both species were extracted with ethanol (95%, and 70%) and H_2O, while fruits of *E. fragilis* were extracted with 70% ethanol. Results showed that 70% ethanol gave the highest percentage of stem extract (21.3%, and 17.7% for *E. alata* and *E. fragilis*, respectively), while fruits gave 35%. The 70% ethanol extract of *E. alata* gave the highest results for TPC, TFC and AA (42.53 mg Gallic acid/ g DE, 87.51 mg Rutin/ g DE, and 70.19 mg $FeSO_4$/ g DE, respectively). On the other hand, 95% ethanol extract of *E. fragilis* showed the highest content of TPC, TFC and AA (32.81 mg Gallic acid/ g DE, 75.44 mg Rutin/ g DE, and 61.68 mg $FeSO_4$/ g DE, respectively). Fruits of *E. fragilis* showed lower results for TPC, TFC and AA compared to stems of both species (14.47 mg Gallic acid/ g DE, 16.96 mg Rutin/ g DE, 38.57 mg $FeSO_4$/ g DE). Stems of *E. alata* showed weak antimicrobial activity against both *E. coli* and *S. aureus* bacteria, but a strong activity against *B. subtilis*. Stems of *E. fragilis* were weak against *E. coli* and showed medium activity against *S. aureus* and *B. subtilis*, while fruits of *E. fragilis* had no activity against all bacterial species. Stem content of both ephedrine and pseudoephedrine were analyzed by a newly developed HPLC method revealing 0.0697% and 0.00411 for *E. fragilis* and 0.00453% and 0.175% for *E. alata*, respectively.

Keywords: - Ephedra, antioxidant activity, antimicrobial activity, total phenolics, total flavonoids, ephedrine, and pseudoephedrine.

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Introduction

Ephedra is a genus of *gymnosperm* shrubs that belongs to the *ephedraceae* family, which includes about 67 species. Ephedra is widespread in many lands, native to Asia, North America, southern Europe and northern Africa[1,2]. Several species of Ephedra have been known for their medicinal properties and were used in traditional medicine. These species include *Ephedra alata, Ephedra fragilis, Ephedra sinica, Ephedra lristanica, Ephedra sarcocarpa, Ephedra strobiliacea, Ephedra procera,* and *Ephedra pachyclada*[3].

Ephedra is one of the plants that attract the attention of scientists in all over the world due to its biological activities. Researches on different Ephedra species extracts showed antioxidant, antimicrobial and antifungal properties. The biological activities of Ephedra come from the photocomposition of individual Ephedra species[4,5]. *Ephedra Sinica* is the most popular species that has been used in China for more than 5000 years[6]. It has been used in some western countries as a dietary supplement[7]. Moreover, it has also been combined with cardiovascular drugs to treat cardiovascular diseases[8].

In addition to ephedrine alkaloids, there are other substances in ephedra, such as polysaccharides, flavonoids, tannins and miscellaneous compounds. More than 145 compounds have been isolated and identified from the genus ephedra and showed anti-inflammatory, anticancer, antibacterial, antioxidant and hepatoprotective activities[5,9].

Due to the presence of these compounds, ephedra has been identified for the treatment of allergies, bronchial asthma, cold, fever, and others[10]. Most of its pharmacological effects have been attributed to ephedrine and analogs[11]. Research have shown that phenolic compounds isolated from *E. sinica* possess antimicrobial activity against both gram positive and gram negative bacteria[12], while antioxidant activity is shown by *E. alata*[13, 14].

A number of side effects for over the counter drugs containing ephedrine analogs has been reported such as epilepsy and loss of concourses[15]. In 2004, the FDA announced the prohibition of dietary supplements containing *E. sinica*[16], which led to difficulties in the use and development of ephedra drugs. In this research, we decided to shed more light on the pharmacological effects of stems and fruits of ephedra. To our best knowledge, *E. fragilis* did not take up space in research in Palestine. Thus, determining its pharmacological properties can help in produce natural sources of potent antioxidants. Additionally, the amount of ephedrine and pseudoephedrine in the stem was determined using a newly developed HPLC-DAD method.

Materials and Methods

Ephedra stems and fruits were collected from Tuqu village (coordinates: 31°38′11″N 35°12′52″E) located southeast Bethlehem-Palestine in September 2022. Identification was confirmed by Dr. Omar Dar Issa (Department of Biology, Bethlehem University, Palestine). All used solvents, reagents and standards were of analytical grade and were purchased from Sigma-Aldrich Company, and thus were used without any further purification. Bacterial species were obtained from The Holy Family Hospital-Bethlehem and the Department of Biology at Bethlehem University. Deionized water was used for analysis and preparation of solutions.

Devices

HPLC Waters Alliance e2695 equipped with 2998 PDA detector (Waters Corporation, MA USA) was used for the determination of ephedrine and pseudoephedrine. Biochem Libra S22 UV-VIS spectrophotometer was used for the determination of flavonoids, total phenolics and antioxidant activity.

Preparation of Plant Material

Stems and fruits of *E. fragilis* and stems of *E. alata* were dried at room temperature, ground and were directly extracted by Soxhlet extractor for two and a half hours using different percentages of ethanol-water solvent (250 ml). Crude extract was obtained by evaporating the solvent by rotary evaporator and it was stored away from direct light at 4ºC.

Pharmacological Properties, Total Phenolic Content, and Total Flavonoids Content

Stock Solution

180.0 mg of each extract was dissolved in 100 mL 50% EtOH. Solutions were reserved for the determination of TPC, TFC and Antioxidant activity.

Total Phenolic Content (TPC)

TPC was determined by Folin-Ciocalteu method[17], which depends on the oxidation of phenolic compounds in the presence of Na_2CO_3.

Analysis test: a standard gallic acid calibration curve was constructed by preparing the dilutions (90-900 ppm). 1.800 mL of Folin-Ciocalteu reagent was added to 40 µL standard/ 100 µL sample. After 5 mins, 1.200 mL of 7.5% Na_2CO_3 solution was added. The solutions were incubated at 27ºC for 60 mins and then measured at 765 nm by an appropriate spectrophotometer.

Total Flavonoids Content (TFC)

Aluminum chloride complex forming assay was used to determine the TFC of the extracts[18]. It relies on complexation reaction with Al (III) after nitration of the aromatic ring by $NaNO_2$ in alkaline medium.

Analysis test: Rutin was used as a standard for the calibration curve with concentrations (5-100 ppm). 300 µL of 5% $NaNO_2$ solution was added to 1500 µL standard/ 500 µL sample. After 5 mins, 300 µL of 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 6 mins.

Then 2000 µL of 1M NaOH solution was added and 2000 µL of H_2O only to sample solutions.

The absorbance of these mixtures was recorded at 510 nm, spectrophotometrically.

Antioxidant Activity (AA)

Antioxidant capacity was determined by FRAP (ferric reducing antioxidant power)[19] method, which depends on the reduction of Fe^{+3} to Fe^{+2} by antioxidants producing blue colored solution.

Analysis test: 1000 µL of FRAP solution and 1000 µL of H_2O were added to 80 µL standard/80 µL sample. The absorbance was recorded after 15 mins at 593 nm, spectrophotometrically.

Antimicrobial Activity

Antibacterial activity of all extracts was studied against *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive) bacteria. An "Agar Well" method was used to test the ability of the extract to inhibit the growth of the bacteria[20]. In this method, three wells were created in the Agar plates of the Muller-Hinton broth[21]: the first of which was for negative control (H_2O), the second was for positive control (Amoxicillin), and the third one was for the sample (extract). 95% ethanolic extract of both *E. alata* and *E. fragilis* and 70% fruits extracts were used for this test. 200.0 mg/3.0 mL DMSO of extract was used for the determination of antibacterial activity. Petri dishes were incubated at 37ºC for 24 hours.

HPLC analysis

Mobile phase composition

The mobile phase consisted of 98% aqueous solution (0.02 M KH_2PO_4 + 3% TEA, pH= 3.0 by H_3PO_4) and 2% acetonitrile. Roc Phenyl-Hexyl HPLC Column (Restek, 150 x 4.6 mm, 3 µm) was used, with a flow rate of 0.7 mL/minute. The PDA range was set from 210 to 400 nm, while the column temperature was set to 25°C. The injection volume was set to 50 µL. All samples were filtered through a 0.45 µL disposable filter before injection.

Standard preparation

Around 50 mg of ephedrine HCl and 50 mg pseudoephedrine HCl were dissolved in 100mL of 20% ethanol. 10 mL was diluted to 100 mL to have a final concentration of 5 mg/100mL of both standards.

Preparation of samples

The preparation of stem samples for both types was done according to the procedure described as "traditional process" by Pi *et al* [22].

Results and Discussion

Extraction

For the leaves of both *E. alata* and *E. fragilis*, results showed that 70% ethanolic extracts exhibited the highest percentage (*E. alata*: 21.3%, *E. fragilis*: 17.7%). Water extraction, on the other hand, showed the lowest percentage (*E. alata*: 14.2%, *E. fragilis*: 6.16%). This may be attributed to the fact that many molecules have both polar and non-polar side groups. So, the presence of water optimizes the effectiveness of the extract. Interestingly, 70% ethanolic extraction of fruits of *E. fragilis* showed extremely higher percentage than all species (35.0%). Results of extraction are shown in table 1.

Table 1. Percentage of extraction

Extract	Result
95% Ethanol (*E. alata*)	20.1 %
70% Ethanol (*E. alata*)	21.3 %
H_2O (*E. alata*)	14.2 %
95% Ethanol (*E. fragilis*)	12.0 %
70% Ethanol (*E. fragilis*)	17.7 %
H_2O (*E. fragilis*)	6.16 %
70% Ethanol Fruits (*E. fragilis*)	35.0 %

Antioxidant Activity, Total Phenolic Content and Total Flavonoid Content

Ephedra alata

For TFC, TPC and AA, 70% ethanol showed the highest content of the compounds and the highest antioxidant activity as illustrated in table 2.

Table 2. TPC, TFC and AA of *E. alata* extract values given per 1 g of dry extract (DE)

Extract	TPC (mg Gallic acid/ g DE)	TFC (mg Rutin/ g DE)	AA (mg $FeSO_4$/ g DE)
95% Ethanol (*E. alata*)	27.26	83.09	58.88
70% Ethanol (*E. alata*)	42.53	87.51	70.19
H_2O (*E. alata*)	32.37	47.86	61.10

The trend of extraction solvent for AA and TPC is in agreement with previous study where both TPC and AA are highest for plant extracted with 80% ethanol.[23]

Phenolic compounds which include flavonoids exhibit a wide range of solubilities in polar solvent and they are more soluble in solvents that are less polar than water[24]. So, it allows higher phenolic compounds to dissolve. Other reason could be related to swelling of the plant matrix. Swelling results from the adsorption of the solute molecules on the hydroxyl and carboxyl groups of cellulose fivers, this favors solvent penetration and the release of soluble compounds into the liquid such as phenols and flavonoids[25]. Thus, giving high AA, which is mainly attributed to presence of the phenolic content in the plant[26].

Ephedra Fragilis

In General, stems of *E. fragilis* showed less content of phenolics and flavonoids and less AA than *E. alata*, while 95% ethanol extract showed the highest results of TPC, TFC and AA, as shown in table 3.

These results indicate that dominant compounds in *E. fragilis* are different in polarity than those exist in *E. alata*. The types of flavonoids and phenolics found in *E. fragilis* tend to dissolve better in 95% ethanol rather than 70%. On the other hand, the highest AA was exhibited by 95% ethanol extract, reflecting the fact that TP and TF are the major determinants of AA.

Although fruits showed the highest percentage of extraction (table 1), but they showed the lowest content of flavonoids and phenolics and exhibited the lowest AA.

Table 3. Values of TPC, TFC and AA of *E. fragilis* extract given per 1 g of dry extract (DE)

Extract	TPC (mg Gallic acid/ g DE)	TFC (mg Rutin/ g DE)	AA (mg $FeSO_4$/ g DE)
95% Ethanol (*E. fragilis*)	32.81	75.44	61.68
70% Ethanol (*E. fragilis*)	32.09	63.64	53.71
H_2O (*E. fragilis*)	25.74	32.11	58.85
70% Fruits extract (*E. fragilis*)	14.47	16.96	38.57

Antimicrobial Activity

As shown in table 4, stem extracts for both *E. fragilis* and *E. alata* had a weak inhibition against *E. coli*, while medium and very weak inhibition were observed against *S. aureus* for *E. fragilis* and *E. alata*, respectively. On the other hand, a strong inhibition for *B. subtilis* was detected when stem extract of *E. alata* was applied, while a medium intensity inhibition took place for *E. fragilis* extract on the same bacteria. Unexpectedly, fruits of *E. fragilis* showed no significant inhibition against all tested bacteria species.

Table 4. Antibacterial activity of *E. fragilis* stems and fruits and *E. alata* extracts against *E. coli*, *S. aureus* and *B. subtilis*.

Extract	*E. coli*	*S. aureus*	*B. subtilis*
95% *E. fragilis* (stem)	Weak	medium	Medium
95% *E. alata* (stem)	Weak	Very weak	Strong
70% *E. fragilis* (fruit)	ND	ND	ND

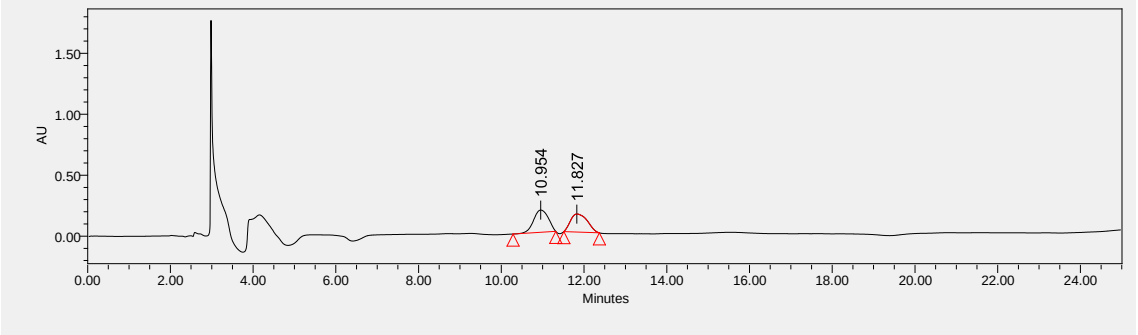
ND: not detectable.

Determination of Ephedrine and Pseudoephedrine content

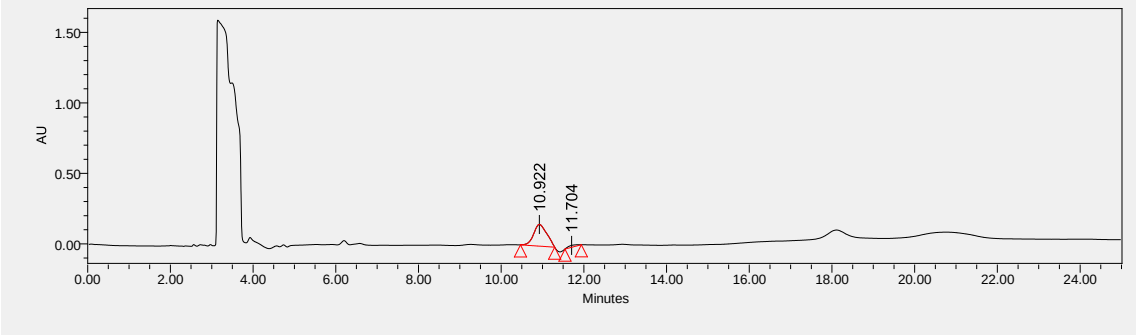
For the separation of the two main components, a phenyl-hexyl column was used, that has a better separation than the usual RP C_{12} or C_{18} columns for such compounds. Figure 1 shows the HPLC chromatograms for both ephedrine and pseudoephedrine standards (A), the stem extracts of *E. alata* (B) and *E. fragilis* (C) samples. Identification of peaks was based on the retention time of the peaks as well as on the UV scan of the diode array detector. The content of ephedrine and pseudoephedrine was calculated using the peak area of their standards (Figure 1) and the results are summarized in table 5.

Table 5: Ephedrine and pseudoephedrine content in studied ephedra species.

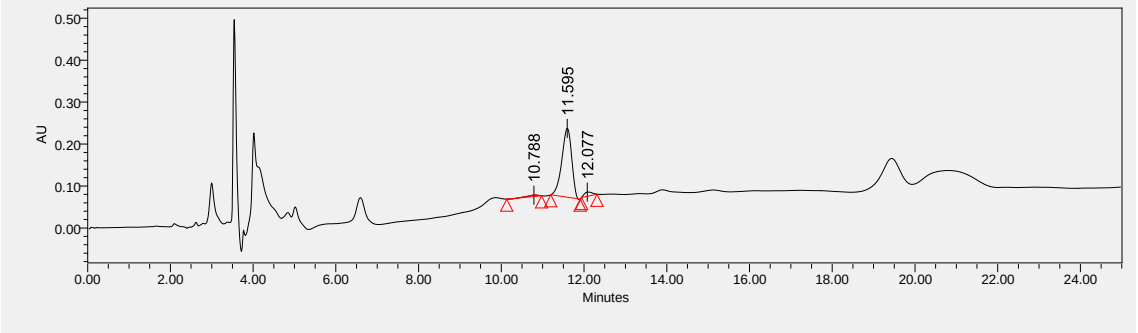
Plant	Ephedrine percent	Pseudoephedrine percent
E. fragilis (stem)	0.0697%	0.00411%
E. alata (stem)	0.0045%	0.175%



A



B



C

Figure 1: HPLC chromatogram for ephedrine (RT ~ 10.9 mins) and pseudoephedrine (RT ~11.8 mins) standard (A), *E. alata* stem extract (B), and *E. fragilis* stem extract (C) detected at 212 nm.

Conclusion

Based on the results, AA in ephedra is mainly correlated to the presence of TPC and TFC in both *E. alata* and *E. Fragilis*. The types of compounds phenolics and flavonoids in *E. alata* are different from that of *E. fragilis*, since in *E. alata* 70% ethanol extract showed the highest results, while for *E. fragilis* 95% ethanol extract showed the

highest results. Fruits of *E. fragilis* showed the highest percentage of extract but with a relatively low content of bioactive compounds. Stems of the two species showed variable antibacterial activity against *E. coli, S. aureus* and *B. subtilus* bacteria, while fruits of *E. fragilis* showed no activity against given bacteria. Relatively low amounts of ephedrine and pseudoephedrine were detected in the stem of both studied species. Further research should be done to identify the bioactive compounds in *Ephedra fragilis* species including fruits to determine the medicinal use of each extract.

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