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Journal of Chemical and Pharmaceutical Research, 2017, 9(6):5-10

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Identification of the Phytochemical Composition of Two Species of *Ephedra* Plants-Traditional Anticancer Herbs-by Gas Chromatography-Mass Spectrometry

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ABSTRACT

A simple, fast, and accurate method was developed for the identification of the phytoconstituents in the methanolic extracts of stems and roots of Ephedra sinica and Ephedra beatona. The developed GC-MS study indicated that the phytochemicals: 1H-Quinolinone, 2-hexadecen-1-ol, 9-octadecenamide and 2-methyl-6-(5-methyl-2-thiazolin-2-ylamino) pyridine were found in the stems extracts in appreciable percent compositions. Stems extracts contain more constituents than roots extracts. It was surprisingly observed that all peaks in Ephedra beatona chromatograms match with those in Ephedra sinica chromatograms; (peak by peak matching) and the major constituents are also similar. The wide use of the two plants in the treatment of several types of cancer and as antidiabetics is the major driving force to study their chemical composition.

Keywords: *Ephedra sinica; Ephedra beatona;* GC-MS; Phytoconstituents; Alkaloids

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INTRODUCTION

Ephedra sinica is a naturally-occurring herb that is used in traditional Medicine. It was primarily used to treat asthma, bronchitis, and hay fever. *Ephedra* is also prescribed for symptoms of cold, flu, cough and fever [1,2]. In Jordan, this herb has two different types: The first type (*Ephedra sinica)* has a dark green stems and the second type (*Ephedra beatona)* has a light green stems with red cycles every 15 cm length period (Figure 1). Both types have no leaves or fruit. These herbs are traditionally used to treat many types of cancer as well as antidiabetics in Jordan which is not well known yet in Jordan and overall the world.

Figure 1: Pictures for the two types of *Ephedra:* A) *Ephedra sinica,* B) *Ephedra beatona*, picked from Tafila city; south Jordan

Ephedra is a member of the Ephedraceae family [3]. Its actions are due to the presence of ephedrine and pseudoephedrine. These two main active ingredient can also be synthesized as a medication and belong to a class of medications called sympathomimetics [4]. It behaves similar to the naturally occurring substance (adrenaline) that

body produces when man feels nervous. Synthetic Ephedrine and pseudoephedrine are used to prevent low blood pressure during spinal anesthesia [5] Both drugs increase blood pressure and act as bronchodilators, with pseudoephedrine having considerably less effect [6]. Synthetic ephedrine compounds are widely used in over-the-counter cold remedies and are regulated as a drug. One of the most important medicinal activities of *Ephedra* plant is its action as anticancer. For example, among many herbal plants used by women with breast cancer, ephedra was the most commonly used and effective plant species in the treatment of breast cancer [7]. Compared to other constituents included in Sho-seiryu-to, the herb *Ephedra* was found to inhibit strongly aminopyrine N-demethylation in rat liver microsomes [8]. Moreover, it is suggested that *ephedra* can serve as a potent candidate for regulation of melanogenesis [9]. This depends on decreasing melanogenic factors (TRP1, MITF, and MAPK) and factors (TNFα, IL-1β, IL-8, and GM-CSF) curing skin cancer and reduce inflammation [10]. *Ephedra* is a traditional medical herb that has anti-inflammatory and anti-oxidant activities. The anti-oxidant activities of the *ephedra* extract was measured in vitro by 2,2- diphenyl-1-picrylhydrazyl (DPPH) and β-Carotene bleaching assays and then examined for possible in vivo hepatoprotective effects of chronic and acute liver failure [11]. The acidic polysaccharides extracted from the stem of *ephedra* were investigated with Box-Behnken design. The method was employed to study the quantitative effects of temperature and time on extraction yield. By solving the regression equation and analyzing 3-D plots, the optimum condition was found at an extraction temperature of 100°C and a time of 3.5 h [12]. The pure polysaccharide ESP-II4 was found to be the main composition of the acidic polysaccharides extracted from *Ephedra* that has *immunosuppressive effect to treat rheumatoid arthritis* [13]. A membrane separation technology including chemical extraction, separation and purification processing was used to extract ephedrine from *Ephedra*. The extraction yield of ephedrine reached 92.49 ± 0.46% with extraction temperature of 80°C and total extraction time of 20 h [14]. The Extract of *Ephedra sinica* was purified and found to have ability to stop the activity of the classical and alternative pathways of complement, and improve neurological outcomes after spinal cord injury and ischemic brain injury [15]. In literature, different analytical methods were used to analyze the phytoconstituents in *Ephedra*. *N*-substituted acetamide glycosides were tested by using spectroscopic examination, after extraction of the amino components in *Ephedra* by ethanol. The structures were elucidated on the basis of multiple 1D and 2D NMR and HRESIMS examinations, and qualitative chemical tests [16,17]. The phytoconstituents in *Ephedra* were measured by using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) by Wang and coworkers [18]. Gas chromatography-mass spectrometry was used to analyze the ephedrine alkaloids simultaneously with its primary metabolites [19]. (GC-MS) study was employed to compare volatile oils in *Ephedra*. The study also described the antioxidant activities of volatile oils extracted from *Ephedra* [20]. Direct ionization-mass spectrometry-based metabolomics analysis method of three medicinal types of *Ephedra* was performed as a direct ionization technique, to provide rapid analysis of samples without sample preparation. So it has been advantageously applied to high-throughput metabolomics analysis [21]. Terpenoids in *Ephedra* were analyzed by gas chromatography-mass spectrometry, and their pure mass spectra were obtained using chemometric methods [22]. NIST library search and retention indices were applied in the identification. The two species of *Ephedra* were chosen in the present study because they are traditionally used to treat many types of cancer in Jordan as well as antidiabetics. This motivates the group to complete this study and identify the phytoconstituents in the two types By GC/MS. Moreover, This work extends the study of the phytochemical compounds in the natural medicinal plants that are investigated in our laboratory [23,24]. The GC/MS technique was successfully used and strong assumptions were built from this method due to the ability for data comparison between the collected results and the documented library adapted with the instrument.

MATERIALS AND METHODS

Plant Materials

The plant samples were harvested from different areas in Tafila city in Jordan. *Ephedra foeminea* is characterized by the red cycles on stems each 15 cm length while *Ephedra sinica* has a homologous stems. Stems and Roots of each plant were separated and used immediately after collection without further treatment.

Reagents

Methanol (HPLC grade) was purchased from Aldrich and used without further purification. Water was distilled by using a distillator type (ELF-2012.

Method of Extraction

Stems and roots of the two species of *Ephedra* (10.0 g each) are accurately weighed and soaked separately in four 50 mL volumetric flasks with methanol. The flasks containing the four materials were manually shaken, and left to

stand for 48 h in dark at room temperature. The methanolic extracts were filtered, collected in new clean volumetric flasks and stored in refrigerator under a temperature of 5°C ± 1 until use.

Gas Chromatography/Mass Spectroscopy

An Agilent Technology gas chromatograph system type 7890 GC equipped with a mass spectrometer type Agilent Technology 5975C Inert MSD triple axes detector was used at a temperature of 250°C. A highly pure Helium gas (99.999%) was employed as the carrier gas. The inlet pressure was 21.5 psi and the makeup gas for the mass spectrometer was highly pure argon (99.999%), at a flow rate of 20.1 mL/min. A column of 5% divinyl 95% dimethyl siloxane, 30 m, 0.25 µm was used. The column oven temperature was programmed as follows: start temperature at 90°C, increased to 280°C with a ramp of 12°C/min, the temperature was held at 280°C for 2 min until elution was complete. The injector temperature was 275°C. After 15 s the split valves were opened for 3 min to purge the injector. All injections (1 µL) were made with a 10 µL Hamilton microsyringe.

RESULTS AND DISCUSSION

The phytoconstituents in *Rubus Fruticosus* [23], *Convolvulus arvensis* [24] and *Artimisia vulgaris* [25] plants were successfully characterized in our lab. These plants were chosen because they have traditionally wide range of therapeutic activity. In this study, two species of *Ephedra* were chosen because they are widely used in tradition medicine to treat many types of cancer in Jordan as well as antidiabetics. This motivates the group to complete this study and find the difference in phytoconstituents in the two species. The gas chromatograph/mass spectrometer was chosen to perform the analysis due to the ability for compound identification beside its acceptable resolution and high sensitivity. Accurate peak area can be calculated to tabulate the present composition for each constituent. Moreover, the extraction method is fast, simple and inexpensive.

Gas Chromatography/Mass Spectroscopy

The best available Chromatographic system in our group is the Agilent GC/MS. The separation was performed by using this modern technique under the conditions described above. The phytocomponents in the methanolic extracts of *Ephedra sinica and Ephedra boatova* were separated and identified. In order to identify the components, a quadrupole mass analyzer was used. The gas chromatograph separates the components and shows a peak for each component in the extract. The mass spectrum contains number of signals. The signal at the highest m/z assigned the mass of the whole molecule. Detailed structural information can be obtained from the fragmentation signals with lower m/z. The base peak appears at 100% abundance. Split mode was used in the analysis and one splitless injection was performed to test whether new components were appeared or not. The mass spectra fragmentation patterns for peaks in the chromatograms in Figures 2 and 3 were compared with the stored library and with other sources for matching components in the extract such as National Institute of Standards Technology (NIST08s), Wiley Registry of Mass Spectral Data's, New York (Wiley 8) and Fatty Acid Methyl Esters Library version 1.0 (FAME library). The acquired mass spectra provided structural information to identify many compounds in the extract mixture (Table 1). By screening the mass spectra and fragmentation patterns with these data, each spectrum matched with one structure with high probability >96% [24-25].

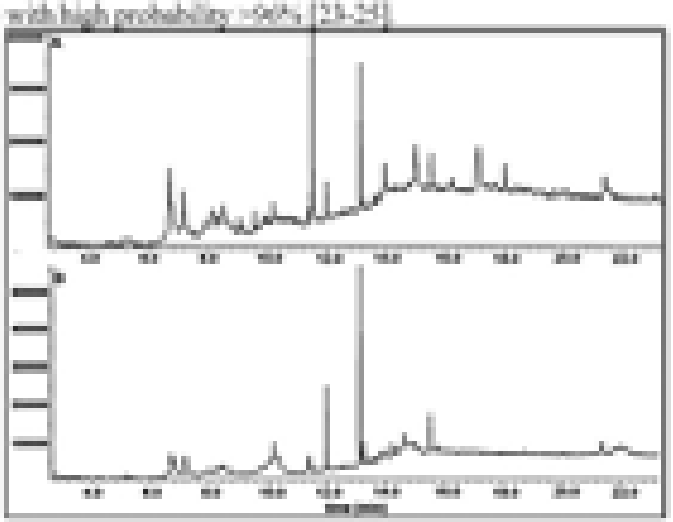

Figure 2: GC/MS chromatograms for the methanolic extracts of *Ephedra sinica*: A) stems extract B) roots extract

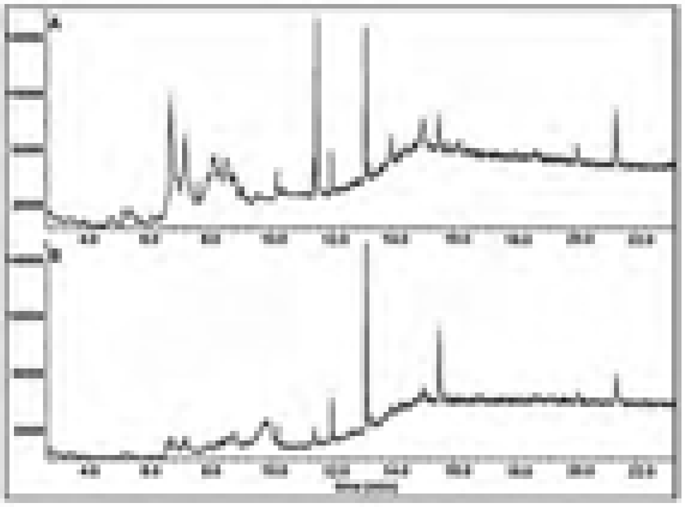

Figure 3: GC/MS chromatograms for the methanolic extracts of *Ephedra beatona*: A) stems extract B) roots extract

Table 1: The detailed information for the components found in *Ephedra sinica* and *Ephedra beatona*

Retention Time (min)	Phytoconstituent	*Ephedra sinica*		*Ephedra beatona*	
		Stems	Roots	Stems	Roots
6.678	2(1H)-Quinolinone	6.56	4.5	10.55	-
6.766	Tetracyclo[4.3.2(4,2).5(6,1).0(3,5)]undeca-7,10-dien-4-ol	3.57	5.23	-	-
7.174	1H-Quinolinone	4.12	4.5	7.02	-
8.485	(3-nitrobut-1-enyl)benzene	1.87	-	-	-
11.522	2-hexadecen-1-ol	37.45	5.23	35.09	3.05
11.988	Hexadecanamide	2.25	5.98	2.11	4.56
13.137	9-octadecenamide	26.09	53.76	31.58	75.76
13.964	2-Ketomethyl-9-tetradecanoic acid	2.25	-	2.75	-
15.029	Cyclohexane, 4-(4-alkylcyclohexyl)-1-pentyl	3.57	-	2.46	-
15.521	4-trifluoroacetoxytetradecane	2.42	21.51	2.46	12.12
16.258	9-octadecenoic acid	1.5	-	-	-
17.129	9,12-octadecadienoic acid, methyl ester	4.5	-	-	-
18.009	Cyclohexane,1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	2.25	-	-	-
21.384	2-methyl-6-(5-methyl-2-thiazolin-2-ylamino)pyridine	2.42	4.5	7.02	4.56

The components with percentages less than 1% are not listed in the table. The listed percentages were calculated as follows: (Area constituent/total area of constituents*100%)

The Major Phytoconstituents

In stems methanolic extract of *Ephedra sinica*, fourteen peaks were detected in the GC chromatogram under the conditions described above (Figure 2 and Table 1). The peaks were individual, sharp, well resolved and readily quantified. All peaks have percent compositions >2%. On the other hand, the chromatogram of stems of *Ephedra beatona* shows nine peaks (Figure 3). It was surprisingly observed that all peaks in Ephedra beatona chromatograms match with those in Ephedra sinica (peak by peak matching). The Compounds with the highest contributions in the two plant species are: 2-hexadecen-1-ol and 9-octadecenamide with high percentages appeared at t_R=11.522 and 13.137 min respectively (Table 1). 1H-Quinolinone appeared in appreciable percent composition at t_R=6.678 min. Roots methanolic extracts consist less phytoconstituents. In the two roots extracts, it is also *clearly observed* that 9-octadecenamide has high percentages with lower percentages of 2-hexadecen-1-ol.

2(1H)-Quinolinone (Figure 4) is member of an amino acid group called alkaloids. These amino acids are found in many plants and have physiological impact on the human body and nervous system. For example quinolones were discovered in 1966 in systematic screening of natural products for anticancer drugs. It was isolated from the bark and stems of Camptotheca acuminata, a tree in China used for cancer treatment in traditional Chinese medicine [26]. Moreover, quinoline compounds are widely used as "parental" compounds to synthesize molecules with medical benefits, especially with anti-malarial and anti-microbial activities. Certain quinoline-based compounds also show effective anticancer activity [27].

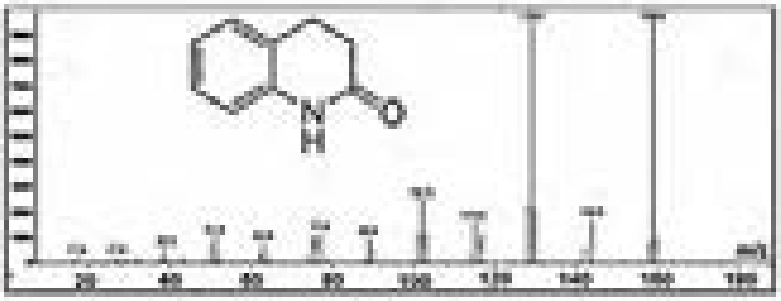

Figure 4: The mass spectrum of 2(1H)-Quinolinone, t_R=4.678 min

9-Octadecenamide (Figure 5) or oleamide was found to be a major component. It is an endogenous substance which occurs naturally in the body of animals to induce sleeping and slowing of blood circulation via endocannabinoid uptake inhibition [28,29].

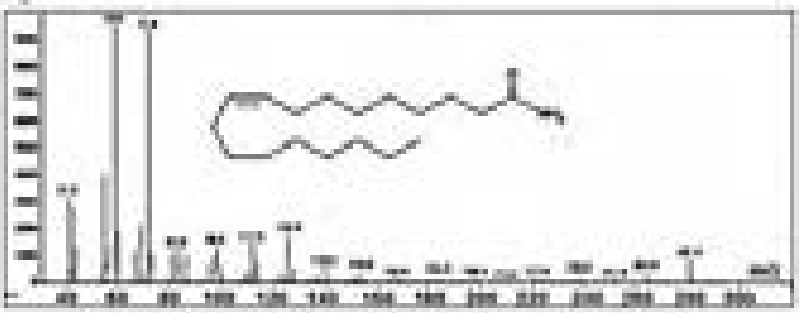

Figure 5: The mass spectrum of 9-Octadecenamide, t_R=13.137 min

2-Hexadecen-1-ol (Phytol) (Figure 6) is an acyclic diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamin E [30] and vitamin K1 [31]. Herbs contain 2-hexadecen-1-ol and prevalent in Korea and china are used in Chinese traditional medicine to treat bacterial infections, hepatitis and tumors and used as diuretic. *In vitro* studies have shown that these herbs exert anticancer activity via caspase-dependent apoptosis [32].

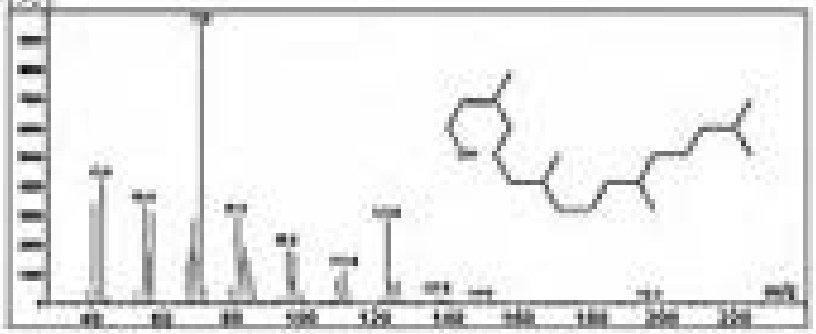

Figure 6: The mass spectrum of 2 hexadecen 1-ol, t_R=11.522 min

2-Methyl-6-(3-methyl-2-thiaoxiran-2-ylamino)pyridine is found in many alcoholic extracts of plants that have anticancer activity [33,34].

Public Applications

The previous data interpret the wide range of biological activity of *Ephedra*. In fact, this study was performed because the two species of *Ephedra* are widely used in tradition medicine to treat many types of cancer and some other symptoms in Jordan. Herein in Tafila city, depending on this information it was surprisingly observed that chewing 2-3 g of *Ephedra sinica* for 15 minutes by people who have hypertension and diabetics, the blood pressure returns normal and glucose level drops down to the normal levels. People who have hypertension and diabetics symptoms stopped using the prescribed drugs such as β-blockers and insulin respectively.

CONCLUSION

The present study confirmed the presence of useful phyto constituents in *Ephedra sinica* and *Ephedra foeminea*, with results comparable with those published earlier and have valuable therapeutic activities especially as anticancers and antidiabetics. These data support the expectations from stems and roots of *Ephedra sinica* and *Ephedra foeminea* as promising sources of useful natural drugs for tumor treatment, hypertension and diabetics. Further in vivo studies and extra purification of the compounds responsible for therapeutic activities are needed.

ACKNOWLEDGMENTS

The authors would like to thank Tafila Technical University for financial support.

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