Interdisciplinary Investigation on Ancient *Ephedra* Twigs From Gumugou Cemetery (3800b.p.) in Xinjiang Region, Northwest China

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ABSTRACT In the dry northern temperate regions of the northern hemisphere, the genus *Ephedra* comprises a series of native shrub species with a cumulative application history reaching back well over 2,000 years for the treatment of asthma, cold, fever, as well as many respiratory system diseases, especially in China. There are ethnological and philological evidences of *Ephedra* worship and utilization in many Eurasia Steppe cultures. However, no scientifically verifiable, ancient physical proof has yet been provided for any species in this genus. This study reports the palaeobotanical finding of *Ephedra* twigs discovered from burials of the Gumugou archaeological site, and ancient community graveyard, dated around 3800 BP, in Lop Nor region of northwestern China. The macro-remains were first examined by scanning electron microscope (SEM) and then by gas chromatography-mass spectrometry (GC-MS) for traits of residual biomarkers under the reference of modern *Ephedra* samples. The GC-MS result of chemical analysis presents the existence of *Ephedra*-featured compounds, several of which, including benzaldehyde, tetramethyl-pyrazine, and phenmetrazine, are found in the chromatograph of both the ancient and modern sample. These results confirm that the discovered plant remains are *Ephedra* twigs. Although there is no direct archaeological evidence for the indication of medicinal use of this *Ephedra*, the unified burial deposit in which the *Ephedra* was discovered is a strong indication of the religious and medicinal awareness of the human inhabitants of Gumugou towards this plant. *Microsc. Res. Tech. 00:000–000, 2013*.

INTRODUCTION

Genus *Ephedra* is a member of Gymnosperm Division Gnetophyta, and is the only genus in its family, *Ephedraceae*. There are ~67 species of the *Ephedra* distributed over a wide area in the dry climates of the north hemisphere, in Asia, the Americas, the southeastern part of Europe and North Africa (Zhao and Xiao, 2009). Among these species, 15 ones as well as four varieties can be found in China with about 10 species in the genus that are used as sources of herbal medicines. In Xinjiang region, 10 species and one variety are naturally available for human use; their distribution in this province covering nearly 66% of *Ephedra* resource in China, marking this region as one of the most important distribution areas of *Ephedra* resources and utilization in the nation.

Human have a long history of *Ephedra* use for medicinal purpose. Pollen of *Ephedra* discovered in Shanidar IV Neanderthal burial site in Iraq over 50,000 years BC (Solecki, 1971) represent the earliest probable evidence of medical and/or ritualistic human use of *Ephedra* (Lietava, 1992; Solecki, 1975). For anatomically modern humans, ancient Chinese may have been among the first to recognize the medicinal value of *Ephedra* (Abourashed et al., 2003). The earliest *Ephedra* pollen were recovered at Banpo site (dating around 4800 to 4300 BC), a Neolithic Yangshao culture in China (Ke and Sun, 1990; Merlin, 2003). *Ephedra* was first documented as an herb medicine for asthma, fever, headache and other pain symptoms in China's earliest Materia Medica (Shen Nong's Herbal Classic) written down during the Han Dynasty(207BC-AD220) (Bensky et al., 2004). Herba *Ephedra* (Mandarin *mahuang*), as the very widely-used herbal medicine in China, in most cases refers to the use of the dried herbaceous stems of three species, *Ephedra sinica* Stapf, *E.intermedia* Schrenk et C. A. Mey and *E. equisetina* Bge (Aonymous, 2010).

Beyond China, a variety of *Ephedra* have also been long utilized for its medication and ritual purposes in several other regions, as is summarized in the review of psychoactive plant by Merlin (Merlin, 2003). In North America, indigenous groups and early European

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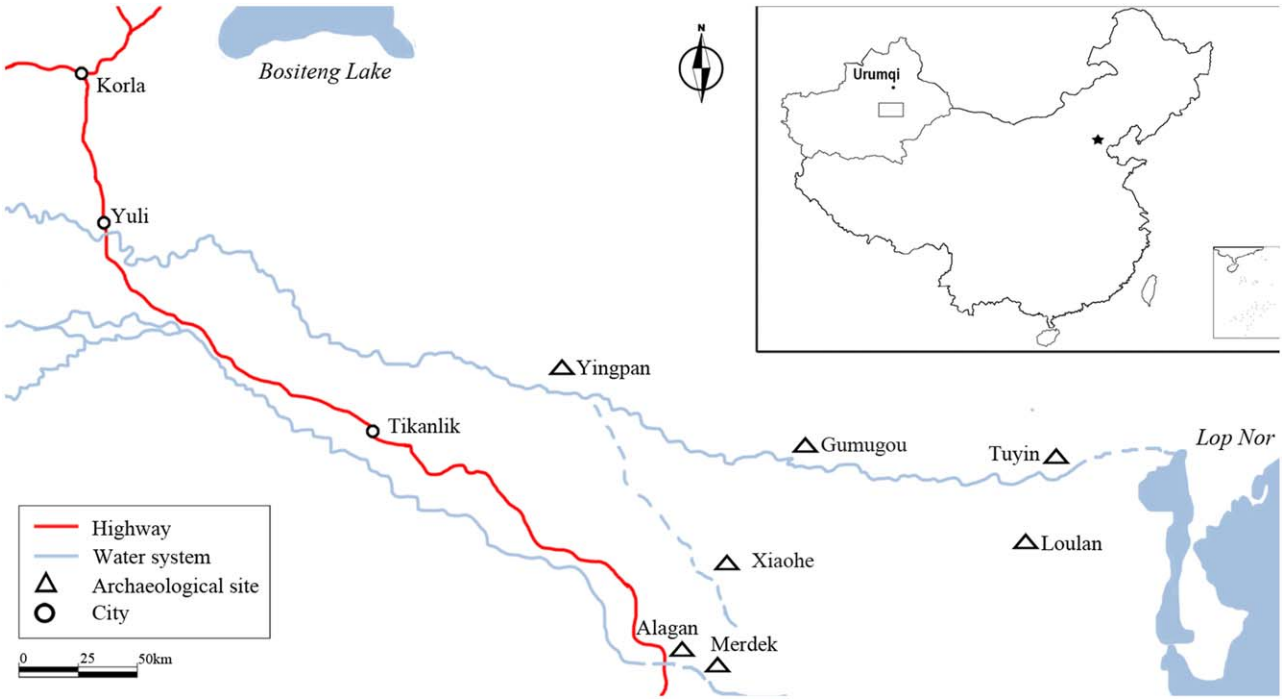

Fig. 1. The location of Gumugou Cemetery in Lop Nor region. [Color figure can be viewed in the online issue, which is available atwileyonlinelibrary.com.]

settlers have a traditional custom of using *Ephedra* plants to make a bitter-tasting beverage, commonly known today in some area as "Mormon tea," to relieve respiratory ailments. In Central Asia, *Ephedra* is one of principle plant that can be possibly identified as the very significant *soma* (or *haoma*) plant or fungus often refered to in the RigVeda of the Indians and the Avesta of the Iranians. According to these ancient Indo-Iranian cultures, *soma* plant was used to make the drink of longevity and drug of immortality (Mahdihassan, 1982, 1983). *Ephedra* was proposed as a candidate specie for soma's origin based on the discovery at Gonur depe, Bactria-Margiana Archaeological Complex (BMAC), a 4000BP Zoroastrian Bronze Age civilization. Remain of Ephedra and Cannabis were recovered from ceramic bowls placed in "sacred fire temple shrine" (Merlin, 2003), suggesting the possibility of making ritualistic drink with the plants (Sarianidi, 1994). Moreover, the ritualistic tradition of *soma* drink consumption seems to also had influence in ancient Greece (Wohlberg, 1990) and is still preserved among Zoroastrians today (Mahdihassan, 1987). Even today, some *Ephedra* species remain as a major herb resource, not only in traditional Chinese medicine, but also in modern pharmaceutical industry.

However, apart from the pollen evidence inside and outside China, very limited solid evidence of *Ephedra* has ever been scientifically reported in any archaeological site worldwide for its medical utilization in ancient times. Only some prehistoric sites in China, all in the Tarim Basin to the west of Lop Nor, claim to have found bundle of *Ephedra* twigs buried along with the dead (Bergman, 1939; Zhang, 2002). In this study, a small bundle of ancient *Ephedra* twigs was selected from one site of this region named Gumugou Cemetery (also called Qäwrighul Cemetery) for microscope observation and GC-MS analysis, in the hope of providing preservation condition of active biomarkers in the plant, as well as implication on its ancient use 3,800 years ago.

Site Description

The Gumugou Cemetery (40°40′35″N, 88°55′21″E) locates in the Taklamakan Desert of Tarim Basin, Ruoqiang County of the Xinjiang Uighur Autonomous Region (see Fig. 1). South to the cemetery is there a river named Peacock River (*Kongque he* in Mandarin), which has dried-up for a long time. During the winter in 1979, 42 graves within the area of 1,600 m^2 were excavated. Radiocarbon dating results shows the cemetery was around 3800BP and thus is one of the earliest Bronze Age cultures in Xinjiang (Han, 2007).

Two types could be divided among the 42 tombs. Type I, locates at the lower layer, covers 36 tombs and therefore is the most representative burial type. A small pack of *Ephedra* twigs has been found in each tomb, all placed upon the right front chest of the dead, revealing a pervasive phenomenon of *Ephedra* burial customs. Type II contains six tombs, each surrounded with seven enclose cyclic annular wooden columns and radialized columns outside the circles (Wang, 1983a). Few burial items have been excavated from this type. The community of the Gumugou Cemetery should be mainly supported by livestock husbandry, with the supporting evidence of horns of goat and cattle. It is

necessary to point out that the absence of ceramics is one major distinguished feature of the site, but large number of small basket woven by grass were excavated (Wang, 1983a,b) instead. The ornate, fine weaving skills reflected from the delicate geometric pattern on these baskets reveal people at that time has already developed relative high level aesthetic pursue on pattern shape and decoration of the basketry, apart from merely the function of that. Another funerary object worth mentioning is pointed felt hats worn by the dead, which are among the earliest felts found in Xinjiang and Central Asia. The unique burial rite of the Cemetery marks it one of the most important archaeological culture in the Tarim Basin for its significant role in the history of human migration and culture communication between the East and the West.

MATERIALS AND METHODS

The ancient *Ephedra* samples were collected from Tomb 79CQM35, while the modern *Ephedra* samples (*E. equisetina* Bge, plant No.01150252) were gathered at the Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences (IBCAS).

Morphological Analysis

The ancient *Ephedra* twigs were observed and photographed under Stereo microscope (Nikon SMZ1000 with camera Nikon DS-Fil), then prepared with a razor, placed on the stubs and sputter-coated with gold using a SPI-MODULE sputter coater. The prepared samples were examined and photographed under a Hitachi-S3700N scanning electron microscopy (SEM) at an accelerating voltage of 25 kV.

Plant descriptions and botanical terms follow the revised English version of the *Flora of China* (http://www.efloras.org/flora_page.aspx?flora_id=2), the structure descriptions are based on the reference of Jiang et al. (2013).

Chemical Analysis

Modern *Ephedra* sample (around 1.0 g) was weighed, ground to powder, and located in a bottom flask with 10-mL deionized water (pH 1–2 with 10% hydrochloric acid). The sample was then extracted under sonication for 2 h at 60°C and alkalized thereafter to pH 11 with 15% sodium hydroxide *(aq)* to free the alkaloids from salt forms. Finally, 15 mL of ethyl acetate was added to extract hydrophobic compounds and filtered. The prepared sample was transferred into autosampling vial for GC-MS analysis.

GC-MS analysis was performed with 7890N gas chromatograph and 5975C mass detector (Agilent Technologies, CA) under MSD ChemStation. Experimental method of the *Ephedra* samples was as follows. For GC part, HP-5 column (30 m × 0.32 mm, 0.25 μm of film thickness) was used with oven temperature from 70 to 150°C at the rate of 3°C min^{-1} (equilibrium 10 min) and 150–300°C at 5°C min^{-1} (equilibrium 20 min). The injector and aux-heater are set at 275 and 300°C, respectively. Helium was used as carrier gas at a flowing rate of 1 mL min^{-1}. The injection split ratio was 10:1. The compounds were identified based on MS spectrum using NIST MS search software (version 2.0f) and AMDIS software within the database of NIST 08 Mass Spectral Library.

Fig. 2. **A**. Broken branchlet of *Ephedra* sp. with node. Scale bar 4 mm. **B**. Broken branchlet of *Ephedra* sp. Scale bar 4 mm. **C**. Stems of *Ephedra* sp. Scale bar 2 cm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

For the ancient sample, the same extraction and GC-MS analysis procedure has been performed, except for the application of splitless injection mode. Blank extraction was simultaneously carried out and tested under the same experimental procedure for contamination control.

RESULTS

Plant Description

As Figure 2 shows, the plant sample have short woody stems, dark brownish with infrequently branches, slender cylindrical and 1–3 mm in diameter. External part has distinct nodes, with bark loosely adhering to the xylem, and a touch of slight roughness. The textile of the plants is light and quite fragile, very easy to be broken with fibrous fracture.

Micromorphology Analysis

Figure 3 shows SEM photographs of radical section of ancient *Ephedra*. Cellular structures of this 3,800-year-old plant preserved well for observation. With bordered pits arraying in one row on the vessels, ephedroid perforation plate has been found on the vessel element. Pits membrane could still be observed from this *Ephedraceae*-featured pattern.

Chemical Analysis

According to Table 1, *Ephedra*-featured biomarkers are identified in both modern and ancient *Ephedra* extract sample. The chromatograph of ancient sample, however, received relatively low concentration, for the possible reasons that many compounds have experienced degradation and sublimation. Therefore, selected ion chromatographs were performed to detect trace content of characteristic chemicals and their degradation outcomes.

Five compounds with significant content have been identified in the total ion chromatograph of modern *Ephedra* sample extract, among which ephedrine is of the highest abundance, followed by benzaldehyde, methyl-pseudoephedrine, tetramethyl-pyrazine, and

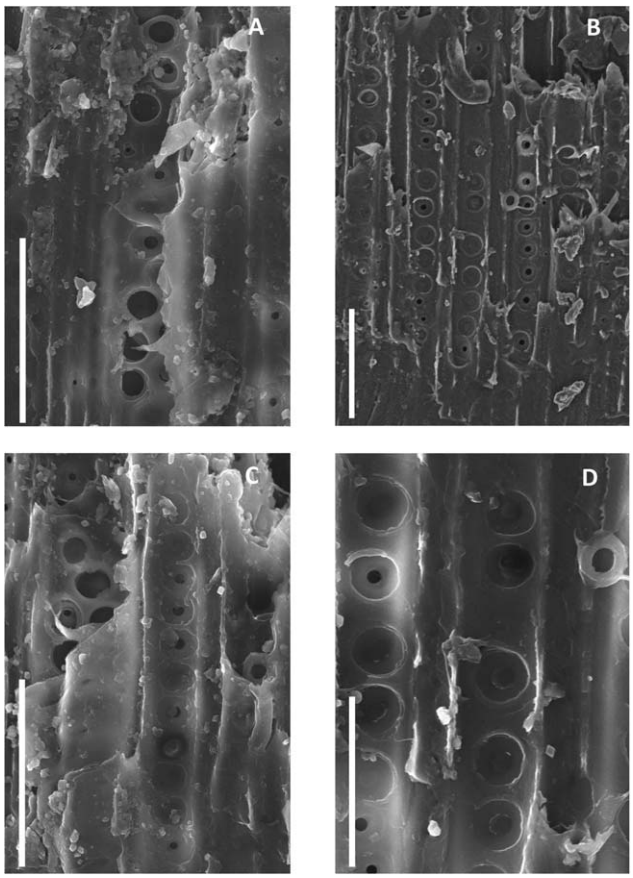

Fig. 3. **A**. Structure of the compound perforation plate of vessel element. Scale bar 50 μm. **B**. Radial section of ancient *Ephedra* showing bordered pits on the lateral wall of tracheary elements. Scale bar 50 μm. **C**. Bordered pits array in one row on the vessel elements. Scale bar 50 μm. **D**. Pit membrane. Scale bar 30 μm.

isothiazole (Fig. 4). Ephedrine and other optical active phenylethylamines, are the most representative constituents of *Ephedra*. According to the TIC of modern sample (Fig. 4), these compounds elute in the time range between 30 and 35 min. In comparison, most chemicals from the same alkaloids family suffered from severe degradation and lost in ancient sample according to its TIC (Fig. 5). Trace of ephedrine was detected in the ancient *Ephedra* extract, yet the peak-noise ratio of ephedrine is too small to reach threshold value. Phenmetrazine, derivative of phenylethylamine was found at 32.8 min (Fig. 5) instead of typical ephedrine-like alkaloids. And finally vanillin, oxidative of vanillic acid, a compound existed in many plants and widely applied in flavor and drug industry elute at 34.7 min (Fig. 5). Compounds were identified by NIST database, with displaying mass spectra (MS) of phenmetrazine (Fig. 6) and vanillin (Fig. 7).

Even with inconspicuous alkaloids identification in ancient sample, some characteristic components have been separated as well. In the first 20 min of the gas chromatograph, where mono- and sesquiterpenes elute, three featured components are identified, with the largest peak of benzaldehyde at 9.7 min (Fig. 8) followed by corresponding peaks of tetramethyl-pyrazine at 15.8 min (Fig. 9), according to retention time sequence.

TABLE 1. Biomarkers detected in modern and ancient ephedra sample extract

	Biomarker existence	
Compound name	Modern sample	Ancient sample
Benzaldehyde	√	√
Benzyl alcohol	√	√
Pyrazine, tetramethyl	√	√
Benzaldehyde,4-hydroxy	—	√
Phenmetrazine	√	√
Vanillin	—	√
3-morpholinone,5-methyl-6-phenyl	√	—
Ephedrine	√	—
(+)-pseudoephedrine	√	—
Phendimetrazine	√	—
Isothiazole	√	—
Methyl-pseudoephedrine	√	—
bacchotricuneatin	—	√

√: peaks respond of the compound; —: no detection.

Benzaldehyde is a featured volatile oil existed in *Ephedra*. In comparison with modern sample, the amount of benzaldehyde in ancient sample shows an obvious decline, indicating that this compound has experienced loss during the burial environment. Yet, this degrading rate of benzaldehyde is much slower than that of alkaloid family of ephedrine. Tetramethyl-pyrazine is another distinguishing volatile oil with the function of asthma relieving, which can be regarded as specific biomarker to trace its biological source. The mass spectra of both volatiles are consistent with NIST database value (Figs. 10 and 11).

DISCUSSION

Herbal *Ephedra* has always been one of most important and commonly used herbal medicine in Chinese history. It is especially effective for diseases involving respiratory system, mostly including cough, common cold, flu, asthma, hay fever, allergy, and so forth. These diseases are generally caused by the evil factor of "wind" according to traditional Chinese medicine theory. Modern medical evidence suggests that *Ephedra* have six main pharmacological effects (Abourashed et al., 2003; Lee, 2011), including: increasing heart rate and elevating blood pressure, constriction of peripheral blood vessel, bronchodilation; mydriasis, central neural system stimulation and urine retention, performed majorly by phenylalkylamines, alkaloids produced by biosynthesis from phenylalanine, as well as other metabolites produced by the plant. The main chemical constituents of secondary metabolites in *Ephedra* include alkaloids, volatile oils and some other kind of components. The GC/MS results show that the alkaloids in *Ephedra* cannot last for a long period of time, esp. phenylalkylamines, with only trace of ephedrine recovered. One reason is that phenylalkylamines are usually sensitive to the PH value and humidity of the environment (Wu, 2008). Buried under the arid gravelly soil surrounding of the site, the alkaloids tend to stay in its chemical prototype rather than salt forms and therefore could be easily lost through volatilization. Another reason that may cause severe alkaloids loss is that most of ephedrine-related alkaloids exist in parenchyma cells of stem pith which decay the quickest and the most complete in the plant. Despite of this, featured components in the *Ephedra*

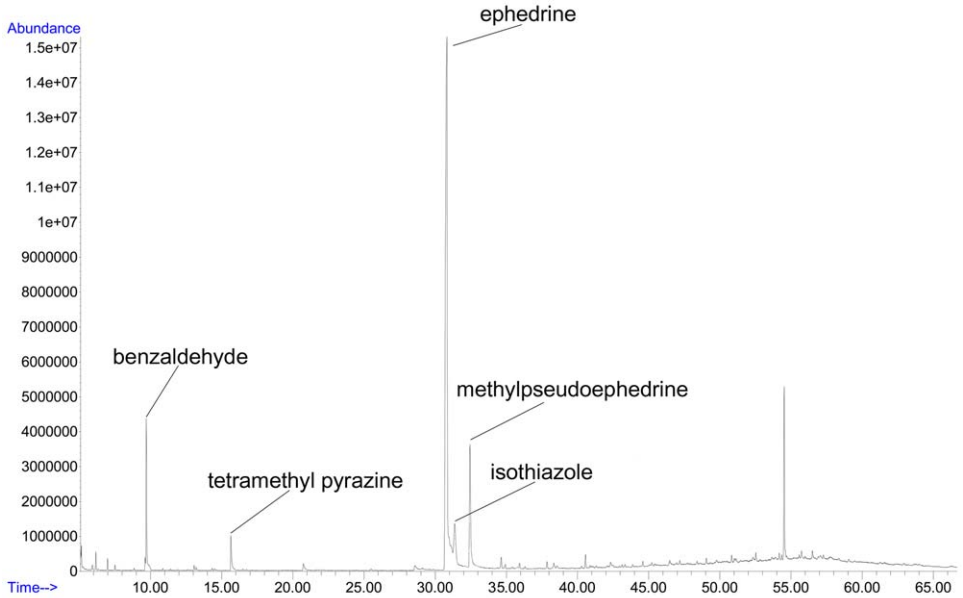

Fig. 4. Complete total ion chromatograph of modern *Ephedra* extract. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

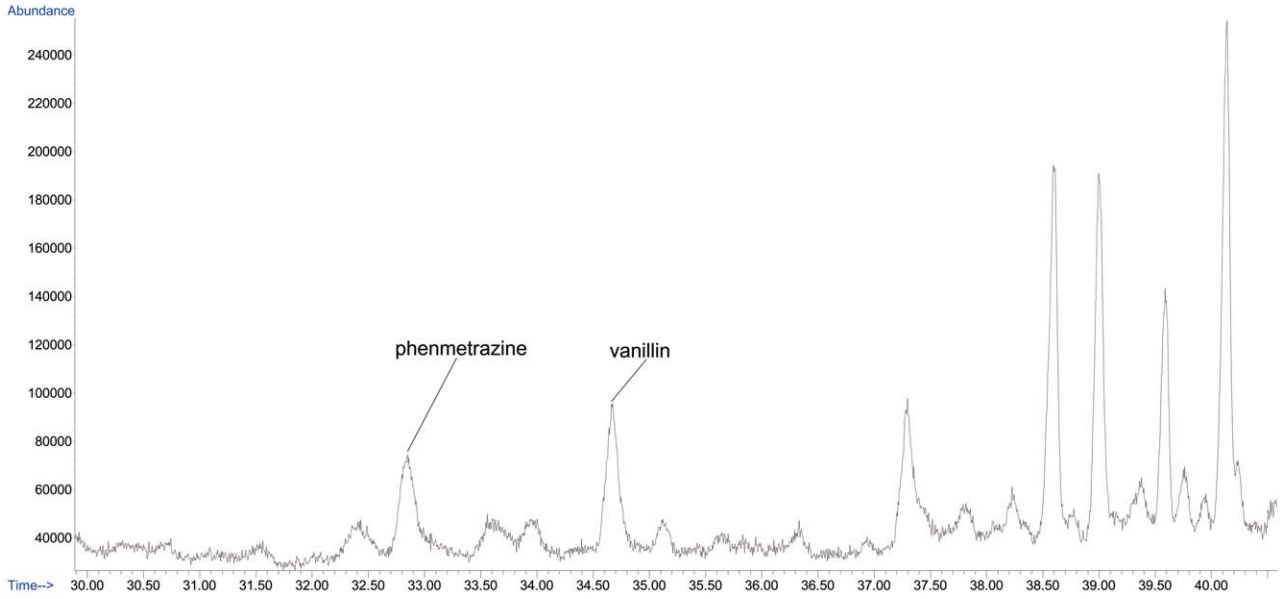

Fig. 5. Total ion chromatograph of ancient *Ephedra* extract (retention time 30–40 min). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

twigs have been successfully separated suggesting that secondary metabolites examination may be a robust candidate for organic *Ephedra* residue identification in some cases.

Furthermore, the morphology evidence can fully convince us that the selected plant remains from the tomb are *Ephedra*. The results presented above suggested that *Ephedra* has been pervasively gathered and fully utilized by ancient population in Lop Nor 3800BP. Yet, what is unique about this site and what is really of interest is the phenomenon that small packs of *Ephedra* are found in each tomb that belongs to tomb type I, indicating that *Ephedra* may not only be treated as regular plant materials but also be of certain association with early medicinal and religious awareness. The reasons are two folds:

On the one side, all the ancient sites that has the phenomenon of buried *Ephedra*, the Gumugou Site,

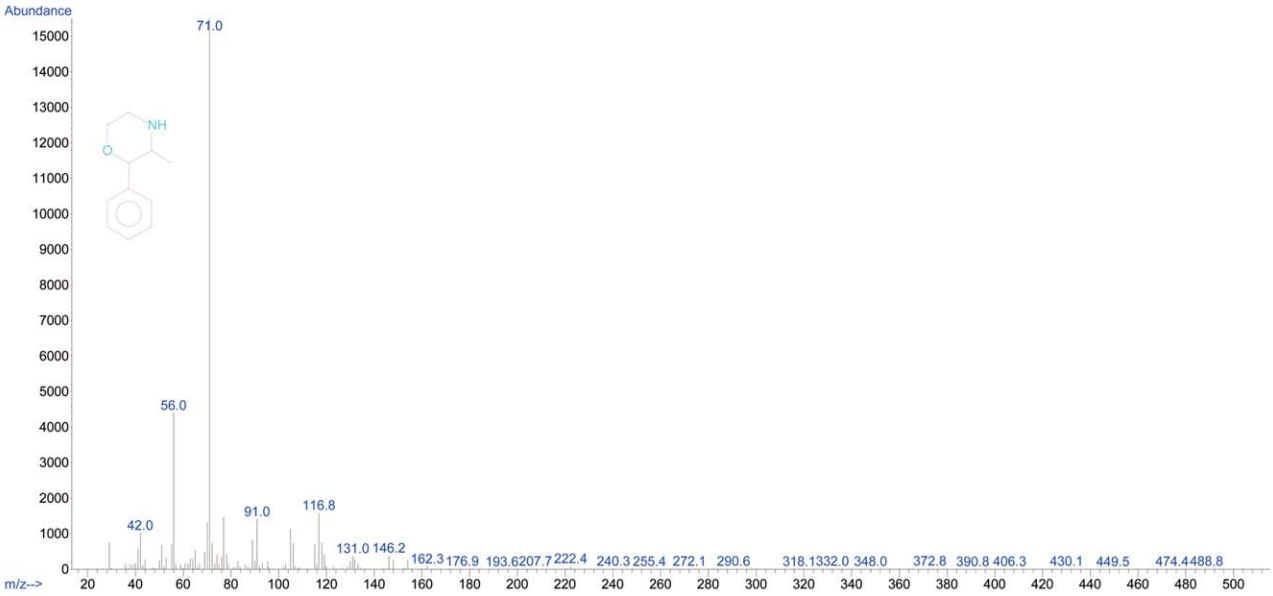

Fig. 6. Mass spectrum of phenmetrazine in ancient *Ephedra* extract. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

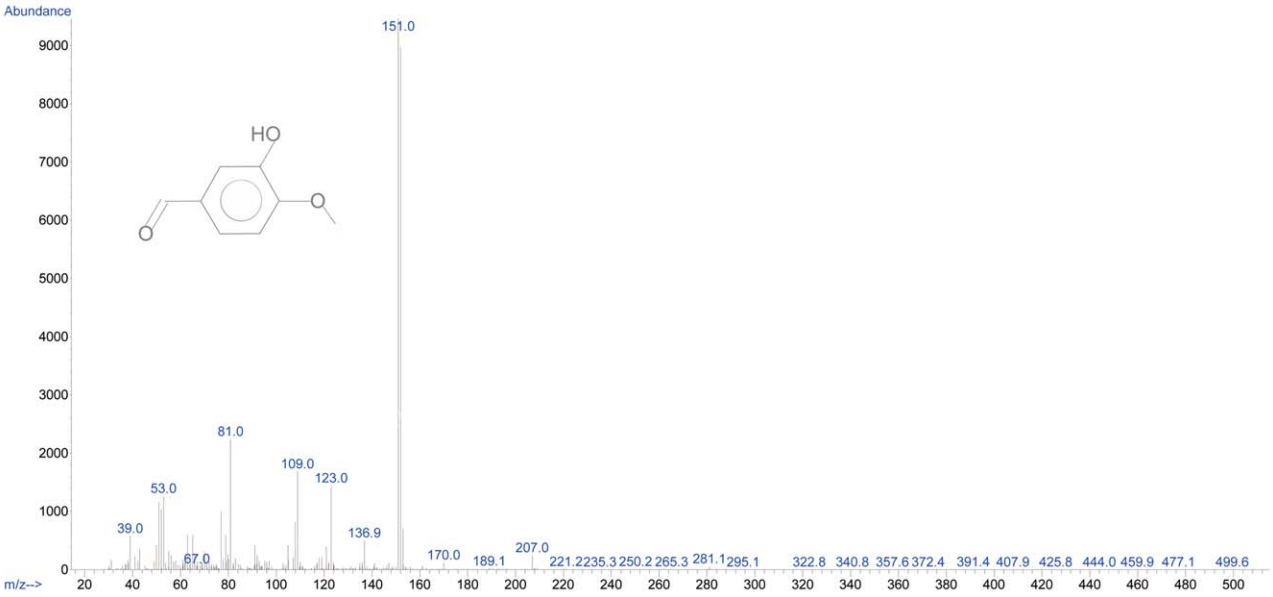

Fig. 7. Mass spectrum of vanillin in ancient *Ephedra* extract. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Xiaohe Site and some other Bronze Age sites, spread evenly among the valley of Peacock River and Tarim River, in the northwest part of Tarim Basin, where luxuriant grassland and oasis located. Though the ancient fertile delta produced by Kongque River provided ancestors a comfortable environment with abundant natural resources, strong wind has always been a constant phenomenon, with evidence of Yardangs scattering everywhere in Lop Nor (Chen, 1936; Yuan and Yuan, 1998). It is particularly easy for Lop Nor settlers to receive respiratory system illnesses; as a result, such extensively popularized *Ephedra* exploitation is not surprising. On the other side, the dry and arid climate in Northwest China make it main habitat for *Ephedra*. In Xinjiang region, more than 20 kinds of *Ephedra* can be easily gotten accessed to, which make

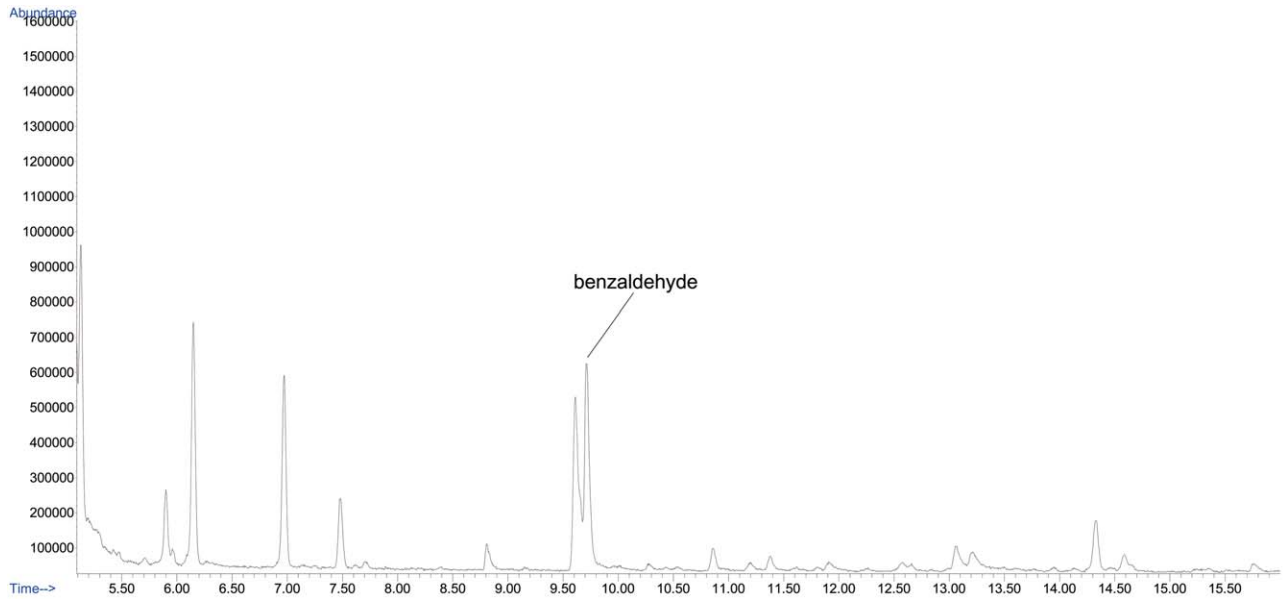

Fig. 8. Total ion chromatograph of ancient *Ephedra* extract (retention time 0–13 min). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

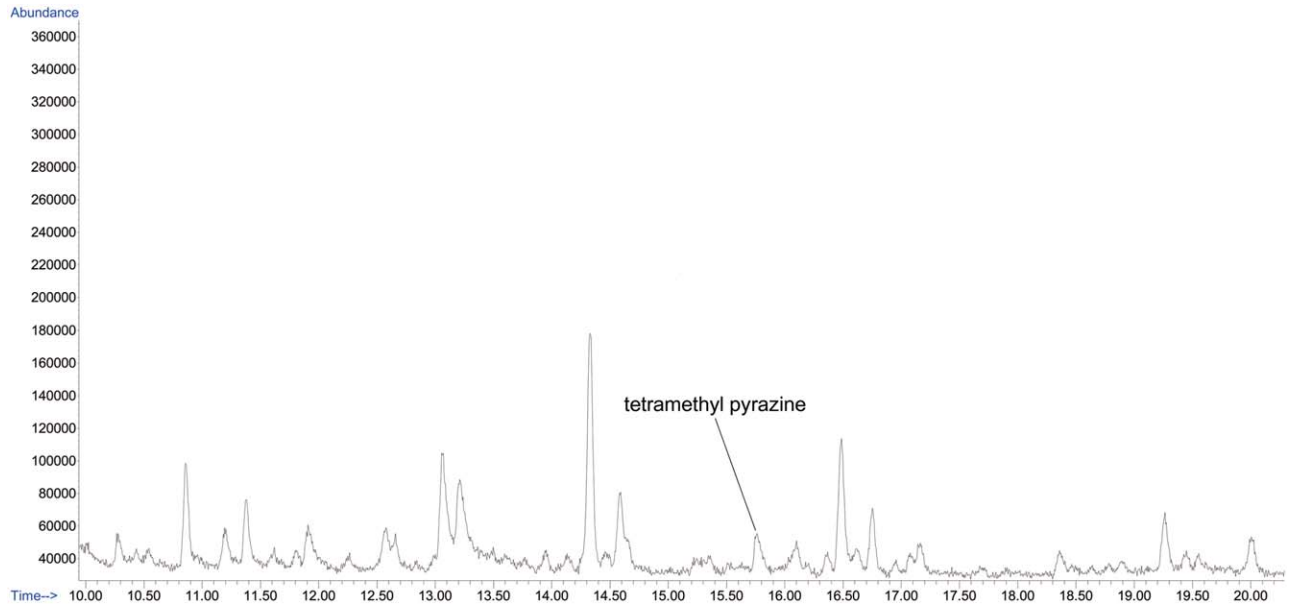

Fig. 9. Total ion chromatograph of ancient *Ephedra* extract (retention time 10–20 min). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Ephedra a most commonly seen plant in Lop Nor; and this is perhaps another major reason that this plant is utilized at such early period.

Another feature that draws our attention is that all the *Ephedra* packs are consistently placed upon the right chest of deceased. One possible explanation given by Bergman (Bergman, 1939) presents that *Ephedra* has an ever-green appearance all year round, giving out strong sense of life in the environment of desert, which made Gumugou people to treat *Ephedra* differently for its sign of energy and life. Beside common efficacy, ephedrine can stimulate central neural system with symptoms such as mental excitement and insomnia. Ephedrine is also the original material for amphetamine. The psychoactive function of ephedrine is quite mild so that it needs a large dose to form obvious psychological effect. As a result, it is not likely that the ancestor use *Ephedra* as spirit hallucinogen

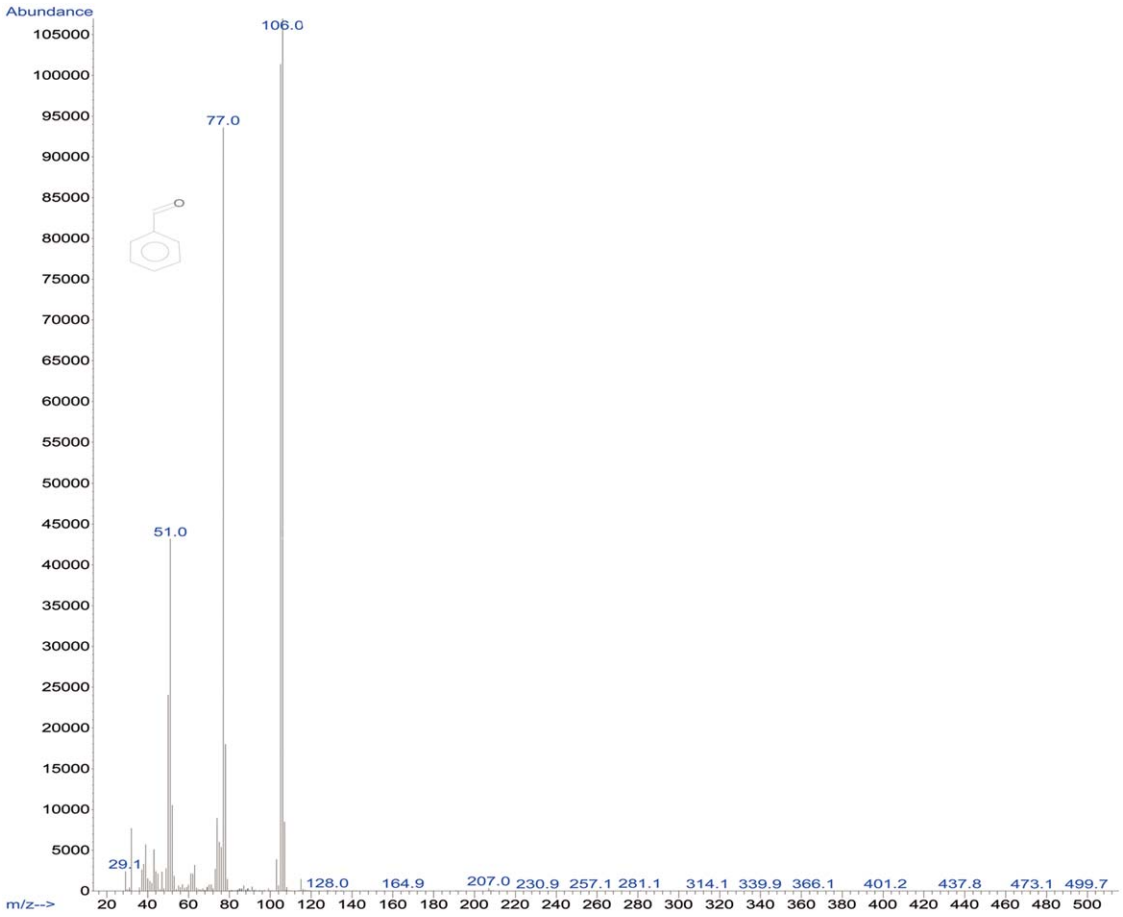

Fig. 10. Mass spectrum of benzaldehyde in ancient Ephedra extract. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

originally, but more for its cure effect and energy boosting function to ensure human's survival and health in the desert (Aili, 2011; Xia, 1997). The early development of medicine and religion are often difficult to separate. Therefore, people from Gumugou treat *Ephedra* majorly as normal medicine but also religion-related wares in view of the placing form and frequency of burial *Ephedra*. Based on the reasons above, the burial formality of *Ephedra* with people in Gumugou Site probably reflect a primitive "plant worship" consciousness towards *Ephedra* by the Lop Nor settlers, which gradually formed the trend of "*Ephedra* worship" culture. The Shanman in Xinjiang region has a historical custom of plant worship, which might has its root in the *Ephedra* worship custom in Gumugou site.

Upon the vast Eurasian Steppe, *Ephedra* utilization has never been an occasional event in human history. Apart from the possibility of Neanderthal's very early awareness on *Ephedra*'s herbal property in Shanidar Cave (Lietava, 1992), *Ephedra* is one of most possible candidate for the representative plant and essential ingredient of the "*soma*/*haoma*" (means the god of plant) documented in the sacred books of Indo-Iranians: the Rigveda and Avesta. *Ephedra* remain recovered from a "fire temple" shrine in south Gonur Tepe, a proto-Zoroastrian civilization site belonging to Bactria–Margiana Archaeological Complex (BMAC), dating back to 2500BC, prompted scholar to confirm *Ephedra*'s function in making ritualistic beverage, reflecting the sacred status of *Ephedra* among Zoroastrian (Bryant and Patton, 2005; Sarianidi, 1993, 1994). The custom of giving the Soma drink to new-born babies for longevity is still preserved among modern Zoroastrians (Mahdihassan, 1983). As the Indo-Iranian tribes migrated and finally settled in Europe and Asia Minor, the original Soma worship in Iran and India spread westwards and found its influence in the Semitic Dionysiac worship (Wohlberg, 1990).

Many archaeological evidences demonstrate that the Gumugou Culture was not isolated. According to the genetic evidence, people at Gumugou have very close relationship with European populations (Cui et al., 2002). The mtDNA sequences falls completely in the range of European lineage and no trace of Asian's has been found (Cui et al., 2004). The molecular genetics results are in consistence with evidence of physical anthropology (Han, 1986), suggesting Gumugou to be a Caucasian community that settled the most east. Moreover, metal artifacts discovered in Gumugou,

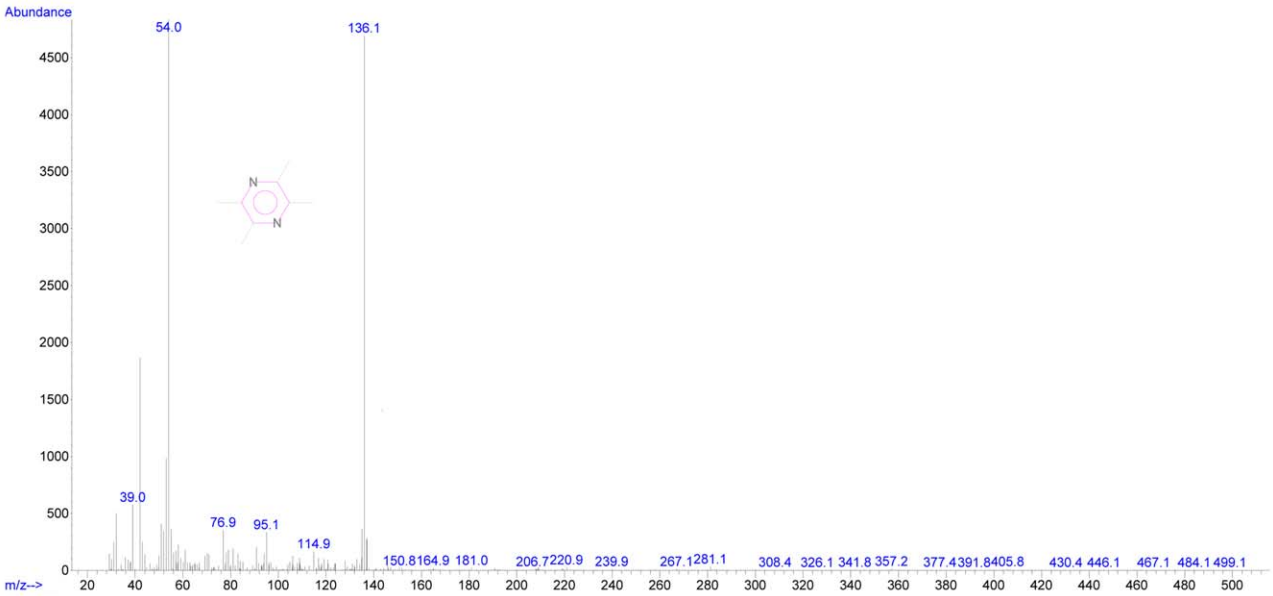

Fig. 11. Mass spectrum of tetramethyl pyrazine in ancient *Ephedra* extract. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

forged of pure copper, own the features of Afanasievo culture from South Siberia (Kuzmina and Mair, 2008). In addition, wheat excavated in Gumugou probably found their way from Central Aisa. All these facts imply that Gumugou had very close communication and relation with other Bronze Age Cultures over the Eurasia Steppe. The finding of *Ephedra* gives proofs for the utilization of this plant during Bronze Age. It is also assumed that close connections between Gumugou and other Bronze Age Culture probably made *Ephedra* worship spread out and gradually rooted in various human cultures that originally generated from the Eurasian Steppe.

CONCLUSION

The plant remains from the Gumugou Site located in Lop Nor, Xinjiang, China, are well preserved due to the arid climate. These remains are identified the oldest example of *Ephedra* through anatomic evidence and active biomarkers through GC-MS protocol, which provide us with significant information on ancient utilization of *Ephedra* in Lop Nor civilizations. This plant has been pervasively gathered and utilized by Gumugou settlers. In the mean time, *Ephedra* packs were unearthed simultaneously with wheat in Gumugou, indicating that ancestors lay the same weight on both to survive, as well as get rid of illness and evil. Although there is no direct archaeological evidence, possible medical function and religious symbol of the plant at the Bronze Age in a way of plant worship can still be inferred, which possibly stimulate the following development of Shamanism among many ethnics in Xinjiang region. The discovery of ancient *Ephedra*, as well as its worship custom, have enlightened us not only on the *Ephedra* development in Chinese medical history, but also help us understand the role that Lop Nor region, where Gumugou locates, played in the cultural exchanges between the East and the West. Our findings successfully present the earliest physical evidence by modern analytical protocols for *Ephedra* utilization on the Eurasia Steppe. From the methodology view, the investigation of GC-MS has provided novel biomarkers to identify the use of ancient *Ephedra*.

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