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High variations of the thebaine concentrations in Iranian poppy (*Papaver bracteatum* Lindl.) from various regions in Iran

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Summary

This paper aims to contribute reliable information to promote the pharmacological utilization of *Papaver bracteatum* by analyzing its wild population in Iran. For this purpose, 27 Iranian poppy specimens from 7 provinces of Iran were analyzed. The highest thebaine concentrations were detected in the capsules, whereas they were relatively lower in the flowering stems. Maximal concentration (more than 53 mg/g d.w.) was present in the *P. bracteatum* plant capsules growing in the Zanus region of Mazandaran province. Surprisingly, the thebaine concentration varies drastically excessively among the tested specimens, ranging from 5 to more than 53 mg/g d.w. Up to now, it is not known whether these drastic differences are due to clonal variations or the differences in the growth conditions, e.g., due to different stress levels of the plants. There is no doubt that the Iranian poppy represents an important alternative source for thebaine, yet, further studies and research are required to promote the farming of this auspicious pharmaceutical plant. In this context, it is of special interest to elucidate the effects of potential genotypes, the impact of the growing conditions as well as seasonal effects.

Keywords: *Papaver bracteatum*, thebaine, HPLC, environmental impact, genotypes.

Introduction

Papaver L. is a large genus comprising about 100 species, which are mainly distributed in the Northern temperate regions (GOLDBLATT, 1974). Based on their morphological characteristics, the genus is subdivided into 11 sections. Among them, the species of the section Oxytona Bernh. are medicinally and economically the most important due to their valuable alkaloid compounds (GOLDBLATT, 1974; KADEREIT, 1988; PARMAKSIZ and ÖZCAN, 2011; SARIYAR, 2002).

Iran is one of the major regions in which many species of the genus *Papaver* are native, and several of them are even endemic. Previously, 25 species of *Papaver* had been described to occur in Iran. However, because of changes in the taxonomic classification of the *Papaver* species (GOLDBLATT, 1974; KADEREIT, 1988), the actual number of *Papaver* species occurring in Iran decreased to 20 (CULLEN, 1966; TAVAKKOLI and ASSADI, 2017). Among these 20 remaining *Papaver* species, five species are endemic to Iran, and the most prominent of them is the Iranian poppy (*Papaver bracteatum* Lindl.) with a well-known beautiful flower and high content of thebaine (Fig. 1). Despite that, the only employed source of thebaine for pharmaceutical purposes is *P. someniferum*.

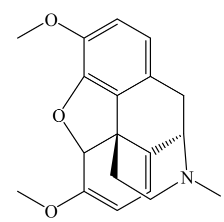

Fig. 1: Chemical structure of thebaine

In the pharmaceutical industry, by various synthetic processes, thebaine is synthetically converted to codeine and two opioid-antagonist, i.e., naltrexone and naloxone and several analgesics such as etorphine, buprenorphine, oxycodone, and oxymorphone (MAZÁK et al., 2019). Due to these various synthetic conversions for producing the mentioned drug, the world demand for thebaine increased in 2019 to about 230 tons (BOARD, I.N.C., 2019). As already outlined, the entire commercially available thebaine is extracted from *P. somniferum*. However, because of its high content of narcotic compounds, such as morphine and narceine, the legally awarded cultivation of *P. somniferum* is restricted to a few countries. Due to the lack of any narcotic components, *P. bracteatum* could be a suitable alternative source for thebaine, which can be cultivated without any legal problems. Accordingly, in countries in which the cultivation of *P. somniferum*, is not allowed, the growing of *P. bracteatum* could cover their demands for thebaine. In addition to pharmaceutical purposes, the plant seed and its extracted oil are a valuable source for the food industries (DUKE, 1973). Moreover, because of its resistance to dryness and high temperature, it can be a good choice for cultivation in arid or semi-arid regions of Iran and other countries with similar situations (NEILD, 1987). It has to be noted that *P. bracteatum* is a perennial plant - a further advantage, since by one-time sowing, the plant can be cultivated and harvested for several years (LEFROY et al., 1999; SCHEINOST et al., 2001). A picture of its habitat is presented in the supplemental material (S1).

Although all these benefits of *P. bracteatum* are widely known, any actual scientific research is mostly missing; the last comprehensive study was published back in the 1980s (SEDDIGH et al., 1982). Accordingly, the question arises why this plant which exhibits such tremendous commercial potential, is not at the center of focus for ap-

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propriate research. A further stimulus for this study is related to the fact that the rare elder works on *P. bracteatum* – at least in part – contradict each other: Qaderi et al. (2019) reported far lower contents of thebaine than previously mentioned by Seddigh et al. (1982) and newly mentioned by Nematolahi et al. (2024). To elucidate the cause of these contrary findings and to generate a solid basis for breeders to select cultivatable varieties for pharmaceutical purposes, we screened many *P. bracteatum* wild-type plants from different habitats in Iran to select genotypes with the highest thebaine concentrations.

Materials and methods

Chemicals

Chemicals and solvents for HPLC analysis were purchased from suppliers as follows: methanol (VWR), ammonium acetate (Fluka, Steinheim, Germany), acetic acid (Fisher Scientific, Loughborough, UK), and triethylamine (Fisher Scientific). Purified water was obtained from the Ultra High Pure Water System (Young Lin, South Korea). Thebaine alkaloid standard was purchased from TEMAD Company (Tehran, Iran).

Collecting botanical samples of Iranian poppy and identifying them

The Iranian poppy specimens were collected from 27 different habitats located in seven different provinces of Iran from 2020 to 2022 (Supplementary, Tab. S1), botanical identification of the Iranian poppy was accomplished according to the key of the botanical book of Tavakkoli and Assadi (2017). In the stage of flowering, the most useful keys for the identification of the *P. bracteatum* species are the number of bracts under its capsules, which generally range from 3 to 5, the color of the flowers, and the shape of the capsules.

Collecting biochemical samples of Iranian poppy

In order to ensure sound and reliable results, all poppy plants were harvested in the fully grown stage, after falling petals and looking yellowish capsules. For collecting samples, an imaginary circle with a radius of 50 m was drawn. For every imaginary circle, three samples and every sample containing 20 flowering stems were harvested. For drying, the harvested samples were stored in a 55 °C of oven.

Sample preparation and extraction

After harvesting the flowering stems, every twenty of them were split into three batches containing capsules, 15 cm below the distal end of the capsule and the rest of the flowering stem. By using a ball mill, the oven-dried plant materials were pulverized to a fine powder. Then, 25 mg of the powdered material was extracted with 1 mL water: methanol (1:3 v/v) adjusted to pH 4 with acetic acid and using an ultrasonic bath for 30 min at 50 °C. The extraction of every sample was repeated four times. The pooled extracts were centrifuged (15,000 g × 10 min). The supernatant was filtered by a cotton filter and used by HPLC. For every 20 flowering stems as one sample, two technical replicates were weighed, extracted and analyzed.

HPLC

HPLC analyses were accomplished by using a binary gradient on a Nucleosil RP-18 (5 µ) 100 Å column. A: Methanol; B: 20 mM $(NH_4)_2SO_3$, containing 0.2% triethylamine. The pH of solvent B was adjusted with acetic acid to 4.0. The HPLC gradient program was at 0 min: 15% A, 85% B; 3 min: 15% A, 85% B; 21 min: 85% A, 15% B. The flow rate and the injection volume were 1 mL/min and 20 µL, respectively. The monitoring of thebaine alkaloid was performed using a UV detector from Agilent (1200 Series) at 280 nm. The quantification of thebaine concentration was based on a calibration curve using five serial dilutions of thebaine standard, ranging from 250 µg/mL down to 5 µg/mL. Sound identification was ensured by the identical retention time and UV spectrum of the Iranian poppy-derived thebaine compared with those of authentical thebaine (Supplementary, Fig. S2 and S3).

Results

The HPLC analysis revealed that the capsules of all tested Iranian poppy samples exhibit quite high concentrations of thebaine (Fig. 2A). In contrast, in the stems, far lower amounts of the alkaloid are present, but – except for the plants from Varvasht of Mazandaran and Jalal Abad of Ardabil – in the distal parts of the stems bearing the capsules, the thebaine concentration is quite higher than in the residual stems (Fig. 2B and C). However, the thebaine concentrations in the *P. bracteatum* plants strongly vary depending on their origin. This high variation of the thebaine accounts for the capsules as well as for the stems (Fig. 2A, B, and C). The highest overall thebaine concentration of 5.3% (d.w.) was determined in the capsules of *P. bracteatum* plants grown in the Zanus region of Mazandaran province. This extremely high value corresponds to near to more than double the mean concentration present in the capsules of the plants from all other regions (Fig. 2A).

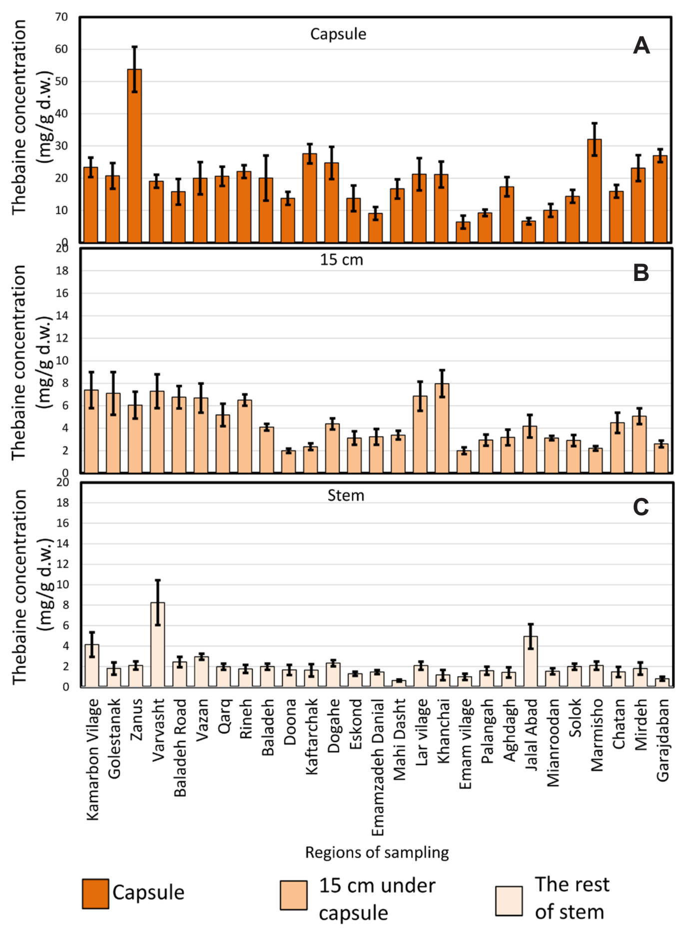

Fig. 2: Thebaine concentrations of *P. bracteatum* in different parts of its flowering stem collected from several habituates in Iran. (A): concentrations of thebaine (mg/g d.w) in the capsules of Iranian poppy; (B): Thebaine concentrations (mg/g d.w) of distal ends (15 cm) of the stems beneath the capsules of Iranian poppy harvested from the different regions; (C): Thebaine concentrations (mg/g d.w) in the rest of flowering stems except their distal ends.

The thebaine concentrations present in the stems of the plants grown in the various areas – except for three regions – are all between one and three mg per g d.w. Only in the plants grown in Varvasht of Mazandaran province, Jalal Abad of Ardabil province, and Kamarbon village of Mazandaran province, the thebaine concentrations in the stems are much higher, i.e., 8.2, 5.0, and 4.1 mg/g d.w., respectively (Fig. 2C). Nonetheless, these elevated concentrations represent only one-tenth of the highest concentration found in the capsules, i.e. 53 mg/g d.w. The highest variation of thebaine concentrations was recorded for the distal stems beneath the capsules, ranging from 2 to 8 mg/g d.w. (Fig. 2B). Surprisingly, there is a correlation neither between the thebaine concentrations in the capsules and the distal stem beneath the capsules nor between the thebaine concentrations in the stems and the distal stems between the capsules.

Discussion

Previous studies presented quite high variations in the concentrations of thebaine in *P. bracteatum*, in particular comparing those of plants collected in different habitats in Iran (Lalezari et al., 1974; Nematolahi et al., 2024; Nyman and Bruhn, 1979; Qaderi et al., 2019; Seddigh et al., 1982; Shafiee et al., 1976). For elucidating the putative causes of these contradictory results, in this study, *P. bracteatum* plants from 27 accessions in Iran were analyzed, including the habitats outlined in the related literature (Tavakkoli and Assadi, 2017). The results indicated that indeed the thebaine concentration in Iranian poppy varies drastically. The highest concentration of thebaine was determined in the *P. bracteatum* plants growing in Zanus in the Mazandaran province, and their maximal value was far higher than the thebaine concentrations in all of the other collected plant samples. Beyond that, it is 50% higher than the concentration reported by Shafiee et al., (1976) for the *P. bracteatum* plants grown in Arya II in Kordestan, which already exhibit about 3.5% of thebaine. It is worth mentioning that the extremely high thebaine concentration determined by Shafiee et al. (1976) had not been reported in any other scientific reports before. Accordingly, the authors attributed these *P. bracteatum* plants to reveal a high commercial value. Consequently, in comparison to these Iranian poppy plants, the *P. bracteatum* analyzed in this study, which exhibits an extraordinary thebaine concentration of 5.3% manifests an even quite higher commercial relevance. Notwithstanding, in analogy to the results of Shafiee et al. (1976) also our results counter the findings of previous studies. Accordingly, the question arises: what are the reasons for these variations? In this context, several states issues have to be considered: apart from putative genetic factors, i.e., different genotypes, above all, differences in the environmental factors, which are known to massively affect the biosynthesis of specialized metabolites, have to be regarded (Barra, 2009; Franz, 1993). Moreover, the time of harvesting is quite important, since the concentration of specialized metabolites frequently differs in plants exhibiting different developmental stages. This especially accounts for substances which are synthesized in one organ (*physiological source*) and translocated in another one (*physiological sink*).

Influence of the various developmental stages

Indeed, any changes in developmental stages are strongly influenced by the particular growing conditions. Consequently, any influence of a certain developmental status is strongly affected by the underlying physiological status, and putative *source-sink*-translocations involved (Ciocan et al., 2023; Pant et al., 2021). Nonetheless, the developmental stage *per se* has an essential impact on the synthesis and accumulation of alkaloids (Shitan and Yazaki, 2007). Based on their studies, the synthesized alkaloids are often highly translocated and accumulated in particular organs of medicinal plants, which are called the 'medicinal part'. In the *P. someniferum* plant, after the translocation of alkaloids in phloem cells, the accumulation of the biosynthesized benzylisoquinoline alkaloids occurs in capsules. In this allocation, transporters, associated with the laticifers, support an apoplastic passage for the translocation of the alkaloids (Ozber and Facchini, 2022), and indeed, this accumulation depends on the specific developmental stage. In analogy, a similar mechanism for the accumulation of alkaloids in capsules of other *Papaver* species has to be expected. This is in accordance with the findings of Seddigh et al. (1982) who showed that the maximum thebaine yield from *P. bracteatum* capsules is obtained when the capsules are harvested five weeks after petal fall when the color of the plant capsules turns yellow. Yet, the central issue for perceiving the specific and intrinsic effect of a certain developmental stage on the synthesis, translocation, and accumulation of specialized metabolites is quite difficult, since we have always to consider that these phenomena interact multifaceted with the multiple impacts of the various environmental factors mentioned below.

Impact of environmental factors

It is well known that the biosynthesis and accumulation of specialized metabolites are strongly affected by numerous environmental factors such as soil conditions, nutrient supply, temperature, or water supply. In this context, in particular, the various stress conditions exhibit the most severe effects on specialized metabolism (Abouzeid et al., 2022; Oladipo et al., 2022; Ramírez-Briones et al., 2017; Selmar and Kleinwächter, 2013a; Yahyazadeh et al., 2023). These stress-related effects not only impact the concentration of specialized metabolites but also influence their spectrum (Abouzeid et al., 2019). Such stress-related consequences had been likewise reported for many alkaloidal plants (Abouzeid et al., 2019; Shukla et al., 2006; Yahyazadeh et al., 2023; Yahyazadeh et al., 2021; Zafar et al., 2020).

As outlined in detail by Selmar and Kleinwächter (2013b), the physiological background of the impact of drought stress on specialized metabolism is well understood: when plants are subjected to drought, the related water shortage induces stomata closure to prevent inadequate water loss. As a result – in addition to the strong decrease in water efflux – also the influx of CO_2 is drastically diminished, whereas the absorption of light energy remains at the actual level. In consequence, under such stress conditions, the plants receive much more energy than they could utilize in the photosynthetic dark reaction, i.e., the Calvin cycle, for the fixation and reduction of CO_2. This excess energy causes a strong over-reduced status, i.e., nearly all molecules of $NADP^+$ are reduced (Selmar et al., 2017; Selmar and Kleinwächter, 2013b). According to the law of mass action, the enhanced concentration of NADPH + H^+ accelerates all reactions consuming the reduction equivalents, including the synthesis of specialized metabolites. This phenomenon is termed "passive shift" (Yahyazadeh et al., 2018; Yahyazadeh et al., 2023). In addition, in many plants subjected to stress, various genes encoding the key enzymes for the biosynthesis of various specialized metabolites are up-regulated – a mechanism denoted as "active up-regulation" (Abouzeid et al., 2022; Yahyazadeh et al., 2018). Both mechanisms, "passive shift" and "active up-regulation" contribute significantly to dissipating the surplus of energy and thereby minimize the stress-related generation of oxygen radicals, which otherwise would destroy the entire photosynthetic system (Selmar et al., 2017; Selmar and Kleinwächter, 2013b).

Furthermore, apart from these both mechanisms, which directly affect the rate of biosynthesis of specialized metabolites, we have to consider that an additional phenomenon may contribute to the stress-related enhancement of the concentration of specialized metabolites: in response to the drought, plant growth is strongly retarded. In consequence – even when the synthesis of specialized metabolites is not affected the concentration of specialized metabolites might be compared to the well-watered control plants, which exhibit a much higher biomass than the stressed individuals do. As a result, even when the **total amount of specialized metabolites** in the stressed plants is the same or even lower than in well-watered control plants, due to massive differences in the reference value, i.e., the biomass, the **concentration of natural products** is significantly higher in the stressed individuals (Paulsen and Selmar, 2016; Selmar et al., 2017). Altogether, there is no doubt that drought stress as well as other environmental factors massively affect the concentration of specialized metabolites - however, the identification of the relevant and underlying mechanism requires a comprehensive and detailed analysis.

Putative differences in the genotypes

Genotypes of a certain plant species may differ by various properties and factors including the manifestation of the specialized metabolism and the environmental impact on it and thus in the production level of specialized metabolites. In this context, several studies outlined such genotypic differences, e.g. two salt-tolerant and -sensitive genotypes of cotton were investigated by Ibrahim et al. (2019): when both cotton varieties had been exposed to drought and salinity stress under greenhouse conditions, the salt-tolerant plants exhibited much higher contents of flavonoids and phenols in comparison to the salt-sensitive plants (Ibrahim et al., 2019). One of the most famous examples of differences in the composition and concentration of specialized metabolites is given by the tremendous variability of peppermint (Kowalczyk et al., 2021; Lu et al., 2022). However, the verification that differences in specialized metabolites of a population of a certain species are the result of different genotypes is quite difficult: due to the numerous interference of the various factors, i.e., clonal variations, environmental and developmental influences on the synthesis and accumulation of specialized metabolites, the problem of a clear differentiation of the particular impact arises. An appropriate approach to vanquish this problem is to cultivate the different genotypes altogether and under various growth conditions. Based on such an approach strategy was realized by ØSTREM (1988), who demonstrated that clonal variations, as well as environmental factors, are responsible for the massive variations in the concentration as along with the spectra of gramine-type alkaloids present in *Phalaris arundinacea*.

Conclusion

The extremely high concentration of thebaine characterizes *Papaver bracteatum* as a valuable source for this highly demanded pharmaceutical alkaloid. Due to the absence of narcotic compounds, such as morphine and narceine, the Iranian poppy could be legally cultivated in countries, in which the cultivation of *P. somniferum* is prohibited. Moreover, because of its resistance to dryness and high temperature, it can successfully be grown in arid or semi-arid regions. As *P. bracteatum* is a perennial, the plant can be cultivated and harvested for several years without further propagation. However, due to the tremendous high variation in thebaine content outlined in this study, it is unavoidable to ensure that in forthcoming cultivation, the plants indeed have the aspired high thebaine concentrations. Accordingly, it is inevitable to identify the reasons for the observed variations in alkaloid concentration. For this, the impact of environmental as well as developmental factors on the biosynthesis and accumulation of specialized metabolites have to be elucidated - and, most importantly, appropriate genotypes of this medicinal plant have to be identified.

As outlined, for such an approach, it does not suffice just to analyze the thebaine content of various populations because of the numerous interferences of environmental and physiological factors in different putative genotypes. As a suitable proceeding, the *P. bracteatum* plants exhibiting the most promising thebaine concentrations have to be cultivated in parallel under the same conditions, and most importantly, these conditions have to be varied with special emphasis on deliberate induction of drought stress.

Declaration of competing interest

It is disclosed and specified that the authors have no potential competing financial interests or personal relationships that could have affected the work described in this paper.

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Supplemental data

High variations of the thebaine concentrations in Iranian poppy (*Papaver bracteatum* Lindl.) from various regions in Iran

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Fig. S1: Habitus of a *P. bracteatum* plant from Zanus region located in Mazandaran Province of Iran.

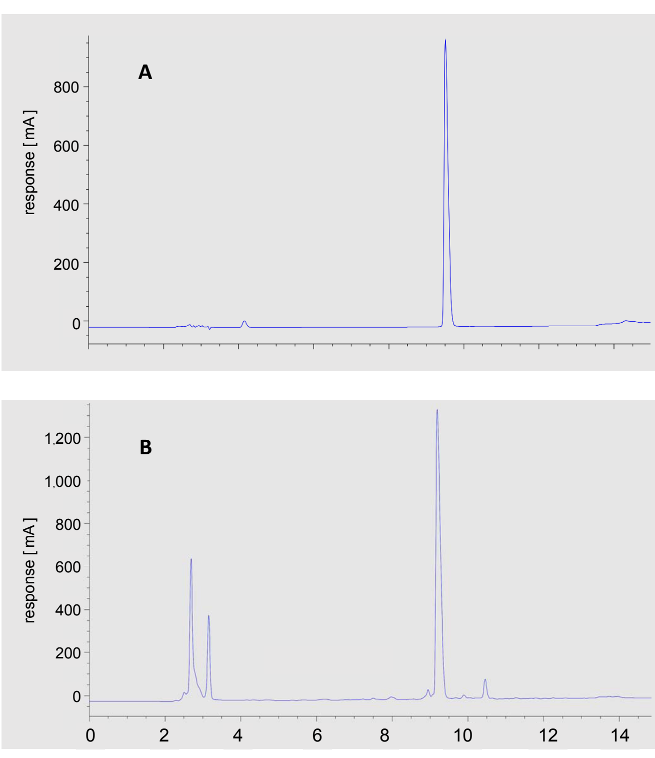

Fig. S2: HPLC chromatogram of thebaine alkaloid standard (A) and plant. sample extracted (B) from Zanus region located in Mazandaran Province of Iran.

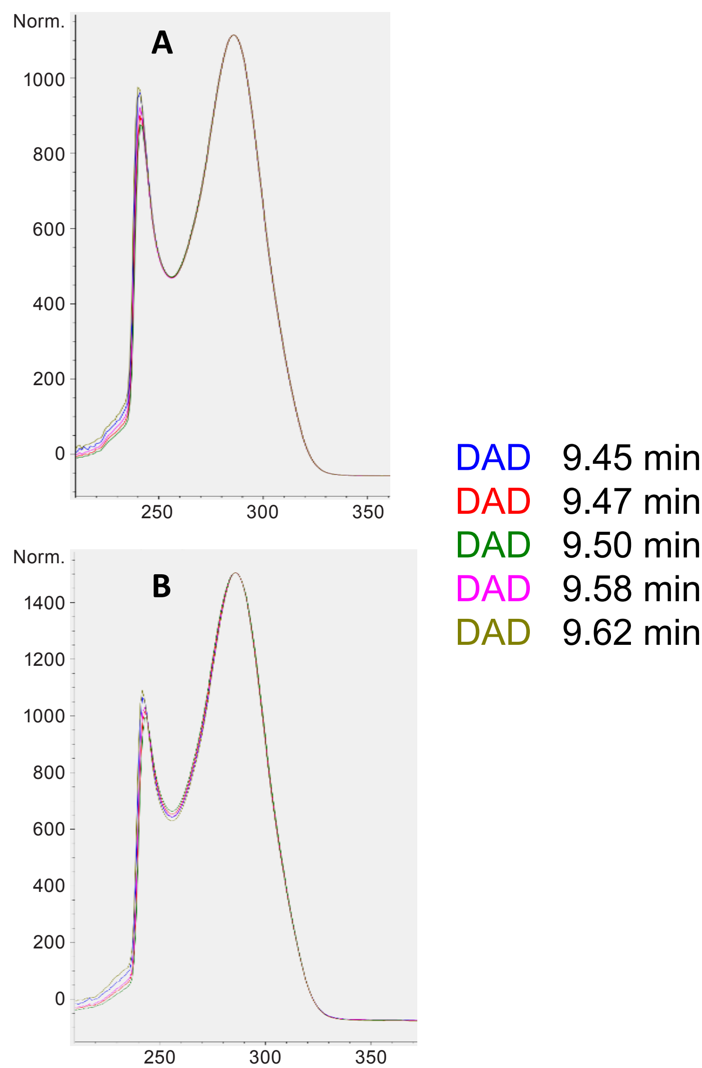

Fig. S3: UV spectra of thebaine alkaloid standard (A) and plant sample (B) extracted from Zanus region located in Mazandaran Province of Iran. For peak purity check, the spectra (recorded by a diode array detector – DAD) of five different time points on the peak of thebaine alkaloid standard (A) and the plant sample (B) were also tested. Every color in each spectrum is one time point of the corresponding peak.

Tab. S1: Origin, elevation, samplers, and date of *Papaver bracteatum* sample harvesting from Iran.

R	Provinces	Name of region	Elevation	Samplers	Date
1	Mazandaran	Kamarbon	2536	Golipour and Yahyazadeh	26 - 7 - 2022
2	Mazandaran	Golestanak	2681	Amini	22 - 8 - 2022
3	Mazandaran	Zanus	1573	Amini	1 - 9 - 2022
4	Mazandaran	Varvasht	2860	Amini	6 - 7 - 2022
5	Mazandaran	Baladeh Road	2571	Golipour and Yahyazadeh	26 - 7 - 2022
6	Mazandaran	Vazan	3072	Golipour and Yahyazadeh	27 - 7 - 2022
7	Mazandaran	Qarq	2430	Golipour and Yahyazadeh	5 - 7 - 2020
8	Mazandaran	Rineh	2713	Golipour and Yahyazadeh	27 - 7 - 2022
9	Mazandaran	Baladeh	2729	Golipour and Yahyazadeh	14 - 8 - 2020
10	Mazandaran	Doona	2500	Amini	2 - 7 - 2021
11	Gilan	Kaftarchak	1780	Amini	22 - 7 - 2020
12	Gilan	Dogahe	1745	Amini	8 - 7 - 2020
13	Gilan	Eskond	1650	Amini	8 - 7 - 2020
14	Qazvin	Emamzadeh Danial	2344	Golipour and Yahyazadeh	3 - 8 - 2022
15	Qazvin	Mahi Dasht	2100	Golipour and Yahyazadeh	22 - 7 - 2020
16	Zanjan	Lar vilage	1943	Moradi	2 - 8 - 2022
17	Zanjan	Khanchai	2220	Golipour and Yahyazadeh	5 - 8 - 2020
18	Zanjan	Emam vilage	1976	Golipour and Yahyazadeh	26 - 7 - 2022
19	Ardabil	Palangah	2340	Bidarlord	12 - 8 - 2020
20	Ardabil	Aghdagh	2000	Bidarlord	12 - 8 - 2020
21	Ardabil	Jalal Abad	2470	Bidarlord	12 - 8 - 2020
22	Ardabil	Mianroodan	2140	Bidarlord	12 - 8 - 2020
23	West Azerbaijan	Solok	1620	Larti	13 - 7 - 2020
24	West Azerbaijan	Marmisho	2770	Larti	28 - 6 - 2020
25	Kurdistan	Chatan	2354	Ajani and Yahyazadeh	9 - 8 - 2020
26	Kurdistan	Mirdeh	2673	Ajani and Yahyazadeh	10 - 8 - 2020
27	Kurdistan	Garajdaban	1810	Golipour and Yahyazadeh	11 - 8 - 2020