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Developmental patterns of phenylpropylamino alkaloids accumulation in khat (*Catha edulis*, Forsk.)

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Abstract

Khat (*Catha edulis* Forsk., Celastraceae) is a perennial shrub that was introduced to Israel by Yemenite immigrants. Khat young leaves are chewed as a stimulant. The main stimulating active principles in this plant are the phenylpropylamino alkaloids (−)-cathinone [(*S*)-α-aminopropiophenone], (+)-cathine [(+)-norpseudoephedrine] and (−)-norephedrine. A novel GC–MS analysis method for the quantitative determination of phenylpropylamino alkaloids and their putative precursor 1-phenyl-1,2-propanedione in khat leaves was developed. We found a marked diversity in the phenylpropylamino alkaloids content and composition in 9 different accessions originated in seedlings and in the commercial cultivar "Mahanaim". The highest 1-phenyl-1,2-propanedione and (−)-cathinone levels occur in young leaves, the part traditionally chewed for its psycho-stimulating properties. Older leaves lack (−)-cathinone but contain the less active (+)-cathine and (−)-norephedrine. Young stems and flowers also contain 1-phenyl-1,2-propanedione, (−)-cathinone, (+)-cathine and (−)-norephedrine. We report the presence of a (−)-cathinone reductase in khat leaves capable of reducing (−)-cathinone to (+)-cathine in the presence of NADPH. We propose that (−)-cathinone is a biosynthetic precursor of (+)-cathine and (−)-norephedrine in khat leaves.

Keywords: Khat; *Catha edulis*; (−)-Cathinone; (+)-Cathine; (−)-Norephedrine; Phenylpropylamino alkaloids

1. Introduction

Khat (*Catha edulis* Forsk., Celastraceae) is a perennial shrub that originated in Ethiopia and cultivated in East Africa, Madagascar and the Middle East (Guantai and Maitai, 1982). Young khat leaves are traditionally chewed in social gatherings to attain a mild euphorical state of mind and stimulant effect among users. Khat was introduced to Israel by Yemenite immigrants and it is still used today in the traditional way and its use is mainly restricted to the traditional Yemenite immigrant's community. Despite its mild narcotic nature, but since the amount of leaves needed to produce an effect and that can be effectively chewed in 1 day is limited (Kalix, 1990), khat is not a controlled plant under the Israeli law. Therefore growing, selling or using khat is a common practice and the plant has gained economic importance to meet the demand of traditional chewers. The khat shrub has two forms, red khat and green khat, also known as white khat. In Israel there is a distinct user's tendency to prefer red khat over the green khat. This raises the question if red khat has higher levels of the psycho-stimulating principles than green khat.

The main psychostimulant principles of khat are the phenylpropylamino alkaloids: (−)-cathinone [(*S*)-α-aminopropiophenone], (+)-cathine [(+)-norpseudoephedrine] and (−)-norephedrine (Fig. 1) (Lee, 1995). (−)-Cathinone is estimated to be one-third as potent as amphetamine and 10 times more potent than (+)-cathine and (−)-norephedrine (Geisshuler and Brenneisen, 1987). It was preliminary found using TLC methodologies that (−)-cathinone mainly occurs in young leaves and may account for 70% of the phenylpropylamino fraction, while fully developed leaves contain no (−)-cathinone but high levels of (+)-cathine and its diastereomer (−)-norephedrine (Guantai and Maitai, 1982; Kalix, 1990). During leaf maturation or during drying and storage, (−)-cathinone is lost and this explains the traditional chewing of only fresh young leaves (Lee, 1995).

Phenylpropylamino alkaloids analysis from plant material is still being developed. A TLC method that is capable of detecting the three alkaloids has been reported, but it is not sensitive

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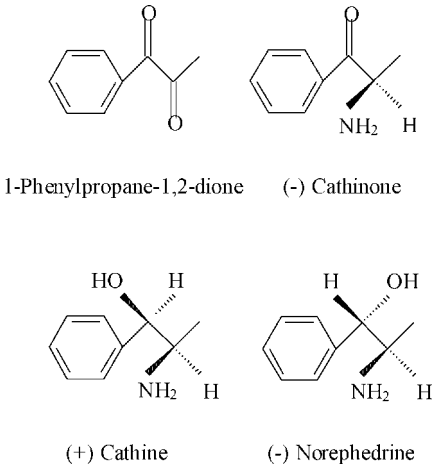

Fig. 1. Khat phenylpropylamino alkaloids and their putative precursor 1-phenylpropane-1,2-dione (PPD).

enough for their accurate quantification (Guantai and Maitai, 1982; Lehmann et al., 1990). Spectrophotometric detection of (−)-cathinone using a copper-neocuproine reagent has been reported (Al-Obaid et al., 1998). This method is not accurate and nonspecific enough, since the reagent stains other ketones present in khat leaves such as 1-phenyl-1,2-propanedione (Fig. 1). Liquid chromatography based methods allow an accurate detection and quantification of khat phenylpropylamino alkaloids (Geisshuler and Brenneisen, 1987). However, the process that is required for sample preparation is time consuming and tedious. This method also requires a relatively large amount of plant material and cannot be easily scaled down. This method is therefore not ideally suitable for the small-scale analysis of a large number of samples. Finally a sensitive GC–MS method for the detection but not quantification of the phenylpropylamino alkaloids has been reported (Ripani et al., 1996). This method is based on acid extraction from plant material then addition of a base followed by partition with organic solvents. Since (−)-cathinone is sensitive to acid treatment, we developed an extraction method that does not require acidic conditions. GC–MS analysis of the extracts was suitable for the small-scale quantitative determination of phenylpropylamino alkaloids in khat leaves This method is also suitable for the quantification of 1-phenyl-1,2-propanedione, a volatile constituent of khat (Abdulsalam et al., 2004) and most probably a metabolic precursor of (−)-cathinone (see below).

The phenylpropylamino alkaloids belong to a relatively minor group of alkaloids within the amino alkaloids that also include the more common phenylethylamino alkaloids such as dopamine and tyramine, found in many families throughout the plant kingdom (Smith, 1976; Kuklin and Conger, 1995). The phenylpropylamino alkaloids (−)-cathinone, (+)-cathine and (−)-norephedrine have been found in only two plant species, in *Catha edulis* (Celastraceae) and at lower levels in *Ephedra gerardiana sikkimensis* (Ephedraceae) (Grue-Sorensen and Spenser, 1994). *N*-Methylation of (−)-norephedrine yields (−)-ephedrine, an adrenolergic compound found in *Ephedra* spp. (Ephedraceae), in *Sida cordifolia* (Malvaceae) and *Pinellia ternate* (Araceae) (Oshio et al., 1978; Shoyama et al., 1983; Khatoon et al., 2005). The *N*-methylation step apparently does not occur in khat (Grue-Sorensen and Spenser, 1994). This step stabilizes the free amino group. In other plants such as *Beta vulgaris* phenyletheylamines and amino acids undergo spontaneous conjugation to aldehydes such as betalamic acid to form betacayanins and betaxanthins (Smith, 1976; Schliemann et al., 1999). It is still unknown what is the chemical mechanism that stabilizes the amino group of the phenylpropylamino alkaloids in khat leaves.

The biosynthetic pathway to phenylpropylamino alkaloids has been studied in *Ephedra gerardiana sikkimensis*. Unlike phenylethylamino alkaloids that are biosynthesized via decarboxylation of amino acids (Kaminaga et al., 2006), phenylpropylamino alkaloids start their biosynthesis by condensation of pyruvic acid and benzoic acid to form the volatile compound 1-phenyl-1,2-propanedione, which undergoes transamination to release (−)-cathinone, which is then reduced to form (+)-cathine and (−)-norephedrine (Grue-Sorensen and Spenser, 1994). Little is known about the biosynthesis of phenylpropylamino alkaloids in khat, but pioneering tracer experiments indicated that the biosynthetic pathway in khat resembles to that found in *Ephedra* (Leete, 1958). Also, the proposed direct precursor of (−)-cathinone in *Ephedra* is 1-phenyl-1,2-propanedione, a compound preliminary identified in the volatile fraction emitted from the khat plant (Abdulsalam et al., 2004). The distribution of 1-phenyl-1,2-propanedione in different plant tissues and its relationship with (−)-cathinone distribution has not been determined. In this study we report the quantitative distribution of phenylpropylamino alkaloids and 1-phenyl-1,2-propanedione in different khat tissues. We also report the extraction of an enzymatic activity from khat leaves capable of reducing (−)-cathinone to (+)-cathine and (−)-norephedrine.

2. Materials and methods

2.1. Plant material and chemical standards

Khat shrubs (*Catha edulis*, Forsk.) were grown in open field conditions under commercial growing practices which include drip irrigation and fertilization, in the Newe Ya'ar Research Center in Northern Israel. The commercial cultivar "Mahanaim" (M) was used for most of the experiments. Additionally, 5 clones each derived from 9 experimental accessions originating in individual seedlings were examined. The plants where 5–6 years old at the beginning of this study. The standards (−)-ephedrine, (−)-norephedrine and 1-phenyl-1,2-propanedione were purchased from Sigma Chem. Co., St. Louis, MO, USA, and (−)-cathinone was synthesized by oxidation of (−)-norephedrine using $KMNO_4$. Human saliva was collected from four healthy male volunteers, combined and immediately used.

2.2. Extraction of khat samples

Two freshly picked leaves where crushed with a mortar and pestle under liquid nitrogen. To the fine powder 3 ml H_2O containing 100 μg (−)-ephedrine as internal standard was added.

The sample was shaken for 30 min at 250 RPM and filtered through one layer of Miracloth (Calbiochem) into an 8 ml glass vial. One and a half ml 1N NaOH where added to the sample in order to de-ionize and retrieve the alkaloids in their uncharged form. Three ml MTBE (methyl *tert*-butyl ether) were added to the sample that was then vortexed for 30 s and centrifuged at $10 \times g$ for 5 min in order to break the emulsion, resulting in a clear fraction separation. The top organic fraction was collected and the process was repeated for a second time, giving a final 6 ml organic extract. Samples were dried by the addition of solid sodium sulfate and then evaporated under a gentle stream of nitrogen to a final volume of 0.5 ml. One microliter was then injected into the GC–MS instrument for analysis (see below).

2.3. GC–MS analysis

The analysis was preformed with an Agilent GC-MSD system model 6890N (CA, USA), interfaced with an Agilent model 5973N Mass Spectrometer. The separation column was a 30 m long Rtx-5 SIL (crosslinked 95% dimethyl–5% diphenylpolysyloxane), having an inner diameter of 0.25 mm and a stationary phase film thickness of 0.25 μm, directly interfacing the mass spectrometer. The gas chromatograph worked in splitless injector mode. Helium was used as the carrier gas with a flow rate of 0.8 ml/min. The injector temperature was 250 °C, and the detector temperature was 280 °C. The oven was operated with the following temperature program: 70 °C was the initial temperature held for 1 min; 10 °C/min up to 125 °C for 5.5 min and then 2.5 °C/min up to 140 °C for 6 min, then 20 °C/min up to 260 °C for 6 min. The temperature of the transfer GC–MS line was 280 °C. A quadrupole mass spectrometer scanned masses in the 41–350 *m/z* range. Compounds were identified by comparison of their MS and retention times to authentic standards.

2.4. Enzyme extraction

The youngest four leaves (approximately 2 g) were freshly picked and crushed in a mortar and pestle under liquid nitrogen, together with 1 g of analytical grade sand and 1 g polyvinylpolypyrrolidone (PVPP). Ten milliliters buffer 50 mM Bis–Tris propane pH 8.0 containing 1% (w/v) polyvinylpyrrolidone (PVP-40) and 1 mM dithiothreitol was added to the samples that were then centrifuged at $21{,}000 \times g$ for 5 min at 4 °C. The resulting soluble enzyme fraction was desalted by gel permeation chromatography on a BioGel P-6 column at a flow rate of 1 ml/min. Fractions of 1.5 ml volume were screened for their protein content using the Bradford reagent (Bradford, 1976), with bovine serum albumin (BSA) as standard. The fractions showing the highest protein level were combined and used in the experiments.

2.5. Enzymatic assay

The enzyme assay reaction mixture for the reduction of (−)-cathinone consisted of 50 mM Bis–Tris propane pH 8.0 containing 1% (w/v) PVP-40 and 1 mM dithiothreitol, 1.5 mM (−)-cathinone, 1.5 mM NADPH, and up to 900 μl protein extract (approximately 90 μg protein) in a total volume of 1500 μl. After an over-night incubation at 25 °C the reaction was terminated by the addition of 1.5 ml 1N NaOH. The alkaloids were extracted twice with 3 ml MTBE. The ether sample was then treated as above and analyzed by GC–MS.

3. Results and discussion

A reliable method for the extraction of 1-phenyl-1,2-propanedione, (−)-cathinone, (+)-cathine and (−)-norephedrine was developed. For that we evaluated different extraction procedures on the same plant material (Fig. 2). There were significant differences between the extraction procedures employed. When the classical alkaloid acid–base extraction procedure was employed, relatively poor results were obtained, The 0.1N HCl–base extraction procedure only extracted 19% of 1-phenyl-1,2-propanedione, 9% (−)-cathinone, 45% (+)-cathine and (−)-norephedrine relative to the water–base extraction procedure.

In order to further evaluate the effect of acidity on the recovery of the related compounds we increased the initial HCl concentration to 1N, followed by a base extraction procedure. In this case, only 9% of the 1-phenyl-1,2-propanedione, 0% (−)-cathinone, 0% (+)-cathine and (−)-norephedrine were extracted relative to the water–base extraction procedure. We conclude therefore that the common acidified-water extraction is detrimental for an efficient recovery of the target compounds observed, although (+)-cathine and (−)-norephedrine were less affected by the acid conditions than (−)-cathinone and 1-phenyl-1,2-propanedione. The most efficient method for the extraction of khat phenylpropylamino alkaloids and 1-phenyl-1,2-propanedione for GC–MS analysis is extraction in water for 30 min then addition of NaOH and separation with MTBE (Fig. 2).

Since young khat leaves are normally chewed as a traditional practice, it was of interest to assess if extraction of the alkaloids with human saliva is efficient. Human saliva is composed of more than 99% water and less than 1% solids mostly

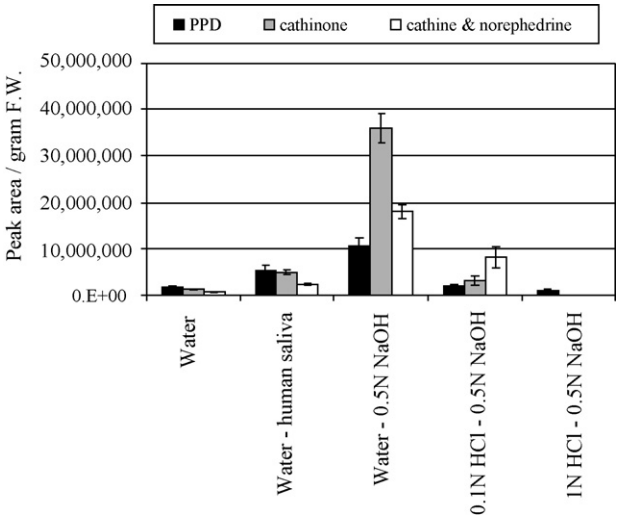

Fig. 2. Different extraction procedures for the GC–MS analysis of phenylpropylamino alkaloids and their putative precursor 1-phenylpropane-1,2-dione (PPD) in khat (*Catha edulis*) leaves. The results are an average of three replicates ± S.E.

proteins and salts, with a buffering capacity of 6.5–7.5 pH due to the content of bicarbonate, phosphate, proteins, calcium, chloride, potassium and sodium (Pedersen et al., 2002). Water followed by addition of human saliva extracted 48% 1-phenyl-1,2-propanedione, 14% (−)-cathinone, 14% (+)-cathine and (−)-norephedrine relative to the water base extraction procedure. Water alone (without the treatment with base) had a pH 6 and extracted 15% 1-phenyl-1,2-propanedione, 4% (−)-cathinone, 4% (+)-cathine and (−)-norephedrine relative to the water base extraction procedure (Fig. 2). It is evident that human saliva has better extraction capability of (−)-cathinone and 1-phenyl-1,2-propanedione than water alone, but lower than extraction with the chemical base (pH 14). This is possibly due to the slightly alkaline nature of human saliva itself which favors some of the alkaloids molecules towards their uncharged form enabling them better partition into the organic fraction.

There are marked differences in the metabolites content and composition between the different khat cultivars (Fig. 3). (−)-Cathinone levels ranged from 6 μg/g F.W. to 368 μg/g F.W., with accessions 71 and 128 having the highest (−)-cathinone content and highest (−)-cathinone percentage in their phenylpropylamino alkaloids fraction 56% and 66%, respectively). The commercial cultivar "Mahanaim" (M) had the lowest (−)-cathinone content and the lowest (−)-cathinone percentage in the phenylpropylamino alkaloids fraction (17%). 1-Phenyl-1,2-propanedione levels ranged from 3 μg/g F.W. to 114 μg/g F.W., with accessions 71 and 128 having the highest content and the commercial cultivar "Mahanaim" (M) having the lowest content. Interestingly, accessions 71 and 128 showed both the highest 1-phenyl-1,2-propanedione (PPD) content, and the highest (−)-cathinone levels (Fig. 3). In parallel, the commercial cultivar "Mahanaim" (M) and accession 25 had both the lowest (−)-cathinone and PPD content, indicating a possible metabolic relationship between these two compounds. (+)-Cathine and (−)-norephedrine levels ranged from 43 μg/g F.W. to 500 μg/g F.W., with accession 8 having the highest content and accession 95 having the lowest content of these compounds.

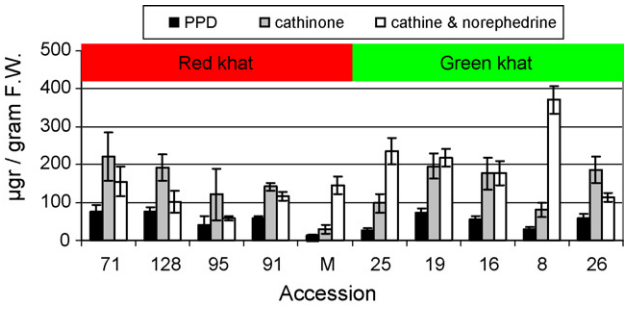

Fig. 3. Composition and content of phenylpropylamino alkaloids and their putative precursor 1-phenylpropane-1,2-dione (PPD) in young leaves (positions 1 + 2) from different khat (*Catha edulis*) accessions. The results are an average of five replicates ± S.E.

Red khat is normally preferred over green khat by traditional chewers. Thus, it was of interest to determine if this preference is reflected in the levels of (−)-cathinone in young leaves. Chemical analysis indicated no major trend in the phenylpropylamino alkaloid composition and content between red and green khat verities (Fig. 3). In average, there is no marked difference in (−)-cathinone and PPD content between red and green khat varieties. Nevertheless, green khat contains in average higher levels

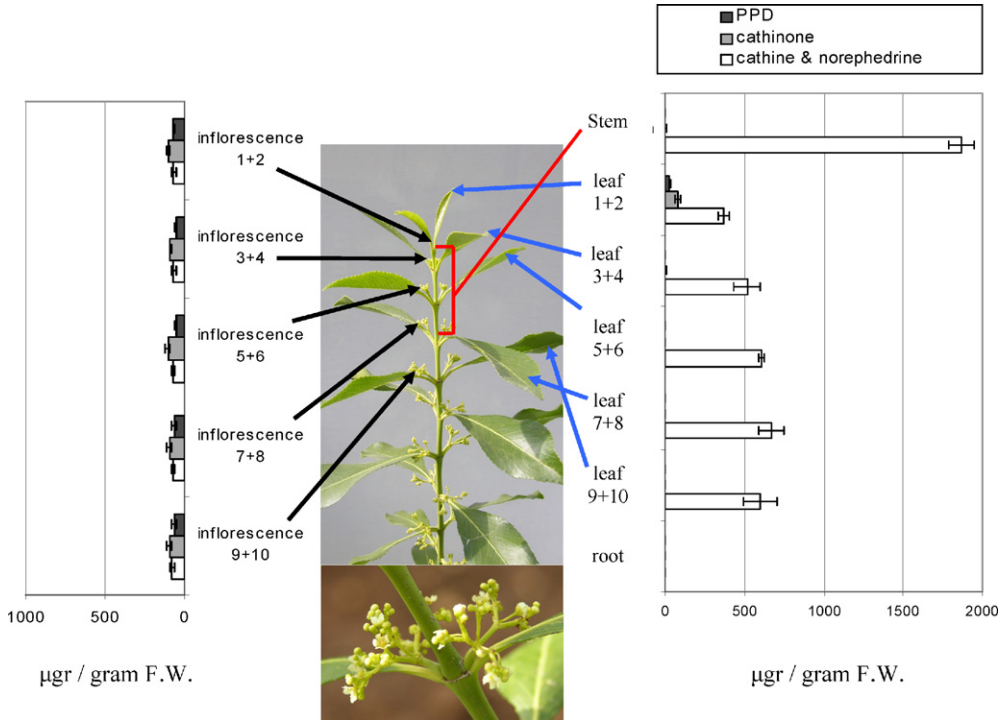

Fig. 4. Relative distribution of phenylpropylamino alkaloids and their putative precursor 1-phenylpropane-1,2-dione (PPD) in different khat (*Catha edulis*) tissues. The results presented were obtained by the analysis of accession 8, but similar results were obtained for other accessions tested. The results are an average of four replicates ± S.E. Numbers represent leaf and inflorescence position from the top. The stem portion was taken from the position indicated in the figure.

of (+)-cathine and (−)-norephedrine than red khat but red khat accessions display in average a higher (−)-cathinone percentage out of the total phenylpropylamino alkaloids fraction (45%) than green khat (35%). Interestingly, both groups (red and green khat) display some degree of diversity in the metabolic content and composition within the group itself, i.e. in each group there are accessions that display high or low alkaloid content. Our results indicate that alkaloid composition and content is genotype specific rather than color group specific, in contrast to the general credence of chewers. This preference of red khat over green khat cannot be explained solely by means of the phenylpropylamino alkaloids composition and content. However it is possible that other yet unknown principles that have some psychostimulant or synergistic capabilities are found in larger quantities in the red khat varieties.

In Israel, only the young leaves are taken in traditional khat chewing, but flowers are sometimes used to prepare a tea with stimulating properties. In other parts of the world, the leaves and stems are also chewed (Abdulsalam et al., 2004). It was therefore of interest to determine the levels of (−)-cathinone in different plant organs, including young and older leaves, stems, inflorescences and roots. (−)-Cathinone is present in young leaves and inflorescences as expected (Fig. 4). The upper parts of the stem also contain low levels of (−)-cathinone, similarly to leaves at positions (3 + 4) and (5 + 6) (Fig. 4). (−)-Cathinone is below detection limits in roots and in leaves at positions (7 + 8) and (9 + 10). These findings correlate well with the common user preference of young leaves, flowers or upper stems for consumption. 1-Phenyl-1,2-propanedione (PPD) has been implicated as a biosynthetic precursor of (−)-cathinone (*en route*) to (−)-ephedrine and (+)-pseudoephedrine in *Ephedra* (Grue-Sorensen and Spenser, 1994), and has been detected previously in khat leaves (Abdulsalam et al., 2004), but its presence in other khat tissues and its developmental patterns of accumulation have not been documented before. PPD is mainly present in young leaves and inflorescences, low amounts of PPD are also found in the upper stem and leaves at position (3 + 4). PPD was not detected in roots and leaves at positions (5 + 6), (7 + 8) and (9 + 10) (Fig. 4). According to these results (−)-cathinone and 1-phenyl-1,2-propanedione show the same distribution patterns during development, present in young leaves and their levels greatly diminish upon leaf maturation. This further corroborates the hypothesis that 1-phenyl-1,2-propanedione may serve as a precursor of (−)-cathinone biosynthesis in khat as found to be the case in *Ephedra* (Grue-Sorensen and Spenser, 1994).

Both (−)-cathinone and 1-phenyl-1,2-propanedione show a juvenile-specific distribution pattern similar to the distribution of naringin in *Citrus* spp. (Bar-Peled et al., 1993). In contrast to (−)-cathinone, the distribution of (+)-cathine and (−)-norephedrine

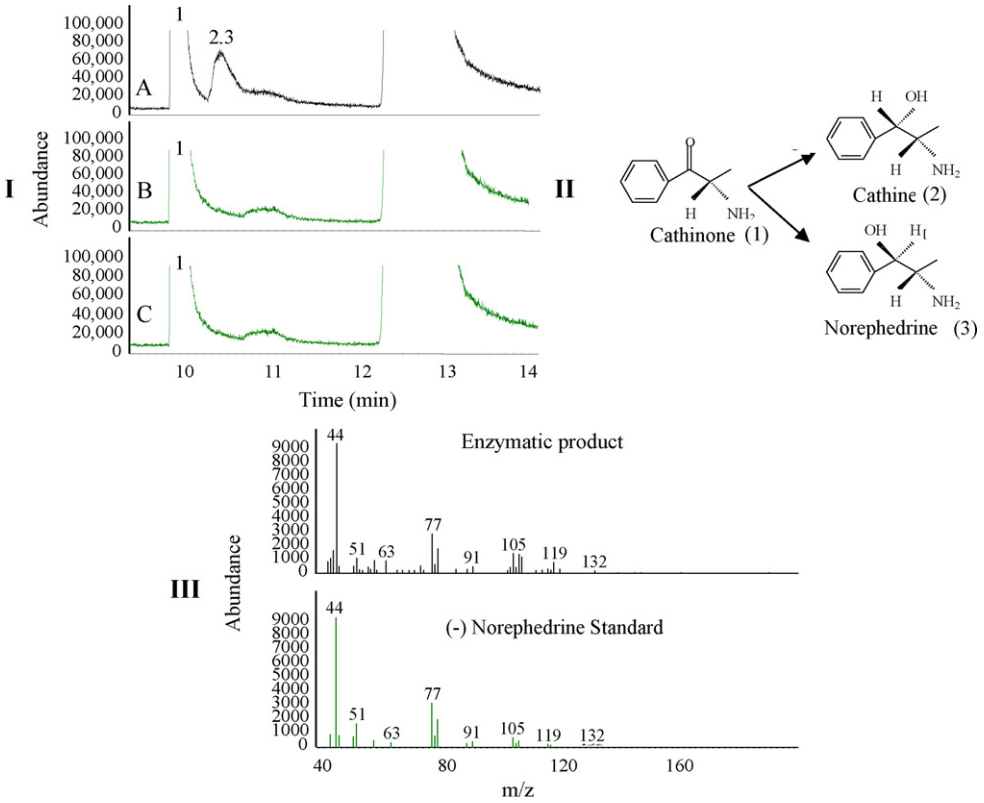

Fig. 5. Cathinone reductase activity in young khat leaves. Cell-free protein extracts prepared from young khat leaves were incubated with the substrate (−)-cathinone (1) in the presence or absence of NADPH under the conditions described in Section 2. Product identification was done by GC–MS according to their retention time (I) and the mass spectrum (III). I: A—complete assay, (−)-cathinone and NADPH as cofactor. B—in the absence of NADPH as cofactor. C—control: no enzyme added. III: the mass spectrum of the biosynthetic product (upper mass spectrum) was compared to the mass spectrum of authentic (−)-norephedrine (3) which is identical to that of its diastereoisomer (+)-cathine (2). II: the molecular structures of the products formed by cathinone reductase activity. The experiment was done with three replicates.

throughout the plant is not juvenile specific. Our experimental analytical conditions did not allow for the full separation of these two diastereoisomers, therefore their occurrence is reported together. All plant tissues examined except the roots exhibit substantial (+)-cathine and (−)-norephedrine levels, with maximum values in the stems and lower values in the inflorescences. Inflorescences have a lower total alkaloid content in comparison to the leaves because they accumulate less (+)-cathine and (−)-norephedrine than the leaves, however inflorescences have a higher (−)-cathinone percentage out of the total phenylpropylamino alkaloids fraction (50%) than the leaves (20%).

The developmental distribution patterns of the phenylpropylamino alkaloids in khat strongly suggest the existence of an enzymatic activity catalyzing the conversion of (−)-cathinone to (+)-cathine and (−)-norephedrine in developing leaves and young stems. Indeed, cell-free extracts derived from khat young shoots (leaves and stems) utilized NADPH as cofactor to catalyze the reduction of (−)-cathinone to (+)-cathine and (−)-norephedrine (Fig. 5). The products formed during the incubations had a retention time and MS identical to those of (+)-cathine and (−)-norephedrine. As shown in Fig. 5, most of the product formed *in vitro* is (+)-cathine but since there is no difference in the MS between (+)-cathine and (−)-norephedrine and due to a lack of baseline separation between the two diastereoisomers under our experimental conditions, we cannot accurately determine the extent of (−)-norephedrine formation in comparison to the formation of (−)-cathine. The enzymatic reduction of ketones to alcohols during leaf maturation has been documented in other plants, such as peppermint (*Mentha* × *piperita*, Lamiaceae), where (−)-menthone is biosynthetically reduced to (−)-menthol by the enzyme menthone reductase 1 or to its stereoisomer (+)-neomenthol by the enzyme menthone reductase 2 (Davis et al., 2005). In opium poppy (*Papaver somniferum*, Papaveraceae) a reductase capable of reducing salutaridine to salutaridinol but not to its stereoisomer 7-*epi*-salutaridinol has been described (Ziegler et al., 2006). In *Hyoscyamus niger* (Solanaceae) tropinone can also be stereospecifically reduced by two distinct tropinone reductases. Tropinone reductase 1 catalyzes the reduction of tropinone to tropine while tropinone reductase 2 catalyzes the reduction of tropinone to pseudotropine (Nakajima et al., 1993). In light of these results we hypothesize that khat leaves contain two reductases that catalyze the conversion of (+)-cathine and (−)-norephedrine, respectively, but further experimentation is required to verify or reject his hypothesis. In any case, the enzymatic activity described here probably accounts for the rapid disappearance of (−)-cathinone from young leaves and the accumulation of the diastereomeric alcohols (+)-cathine and (−)-norephedrine in mature leaves.

4. Conclusions

In conclusion, khat is a little studied but commonly used plant in several parts of the world, while a controlled and controversial plant in other locations (Szendrei, 1980). We have developed a reliable rapid small-scale quantitative analysis method for the analysis of phenylpropylamino alkaloids and their putative precursor PPD from khat leaves using GC–MS. Using this methodology, a marked diversity in the metabolites content and composition in 9 different accessions and the commercial cultivar “Mahanaim” was found. The detailed quantitative distribution of phenylpropylamino alkaloids and 1-phenyl-1,2-propanedione in different khat tissues was reported, and a biochemical mechanism for the conversion of (−)-cathinone to (+)-cathine and (−)-norephedrine in khat leaves was proposed. This work is a contribution to our understanding of the patterns of alkaloid accumulation in khat in attempts to rationalize some of the ethnobotanical practices associated with the use of this plant.

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