Naturally Occurring Norephedrine Oxazolidine Derivatives in Khat (*Catha edulis*)

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- norephedrine
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- phenyloxazolidine alkaloids
- GC-MS

Abstract

Khat (*Catha edulis* Forsk.) is a perennial shrub whose young leaves are chewed for their psychostimulating and anorectic properties. The main active principles of khat are believed to be the phenylpropylamino alkaloids, primarily (−)-cathinone [(*S*)-α-aminopropiophenone], (+)-cathine [(1*S*)(2*S*)-norpseudoephedrine], and (−)-norephedrine [(1*R*)(2*S*)-norephedrine]. GC-MS analyses of young leaf extracts indicated the presence of two oxazolidine derivatives, 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine. To ascertain the chemical identity of these compounds, we synthesized the putative compounds by condensation of norephedrine and acetaldehyde or *trans*-2-hexenal, respectively. Spectroscopic analyses (GC-MS, NMR) of the structures of these synthetic compounds showed them to have identical retention indexes and mass spectra characteristic to 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine. Marked differences in the ratios between each of these two norephedrine oxazolidine derivatives and total phenylpropylamino alkaloids were found among thirteen different khat accessions further indicating polymorphism in alkaloid ratios and content in *C. edulis*.

Introduction

Khat (*Catha edulis* Forsk., Celastraceae) is a perennial shrub that is cultivated in parts of Africa and the Middle East [1,2]. The main active principles of khat are believed to be (*S*)-cathinone, (1*S*)(2*S*)-norpseudoephedrine and (1*R*)(2*S*)-norephedrine, three phenylpropylamino alkaloids that are central nervous system stimulants, mild euphoriants, and anorectics [3]. In general, phenylpropylamino alkaloids mimic the action of adrenaline and cause the release of endogenous cathecholamines from post-ganglionic sympathetic fibers [4]. (*S*)-cathinone is estimated to be one-third as potent as amphetamine and ten times more potent than (1*S*)(2*S*)-cathine and (1*R*)(2*S*)-norephedrine [1,5]. The metabolic precursors of the phenylpropylamino alkaloids occur mainly in young khat leaves. Fully developed leaves contain only trace amounts of these two metabolites but have higher levels of (1*S*)(2*S*)-norpseudoephedrine and its diastereomer (1*R*)(2*S*)-norephedrine as compared to young leaves [1,6–8].

Phenylpropylamino alkaloids constitute a relatively minor subgroup within the amino alkaloids [9–13]. (*S*)-Cathinone, (1*S*)(2*S*)-norpseudoephedrine, and (1*R*)(2*S*)-norephedrine have been found in only a few plant species, such as *C. edulis* (Celastraceae) and *Ephedra gerardiana sikkimensis* (Ephedraceae) [14]. Interestingly, the phenylpentenylamine alkaloid merucathine, structurally resembling cathine, has also been detected in *C. edulis* [9]. *N*-methylation of (1*R*)(2*S*)-norephedrine and (1*S*)(2*S*)-norpseudoephedrine yields (1*R*)(2*S*)-ephedrine and (1*S*)(2*S*)-pseudoephedrine, respectively, adrenolergic compounds found in most, but not all, *Ephedra* spp. [15]. A related compound identified in *Ephedra* species include 3,4-dimethyl-5-phenyloxazolidone (ephedroxane), a heterocyclic oxazolidone derivative of ephedrine that possesses anti-inflammatory activity [16]. Other oxazolidine derivatives such as 2,4-dimethyl-5-phenyloxazolidine and 2,3,4-trimethyl-5-phenyloxazolidine are possibly formed from the spontaneous conjugation of ephedrine and acetaldehyde [17]. Phenylpropylamino alkaloid-derived oxazolidines are pharmacologically active stimulating prodrugs that readily hydrolyze in aqueous systems to release the pharmacoactive phenylpropylamino alkaloid

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from the conjugated aldehyde [18–20]. The above-described *N*-methylation, oxazolidine, and oxazolidone derivatives of phenylpropylamino alkaloids found in *Ephedra* could serve as a secondary stabilization mechanism for the highly reactive free amino group. In *L. williamsii,* a total of 14 conjugates derived from the active principle mescaline and various Krebs cycle components were discovered [12,13]. In *A. berlandieri* and *A. rigidula,* two other unique phenylpropylamino alkaloids have been identified, amphetamine and its *N*-methylation derivative, *N*-methylamphetamine [21,22]. However, to date, the chemical mechanism that stabilizes the amino group of the phenylpropylamino alkaloids present in khat leaves is still unknown. We have previously speculated about the existence of such a mechanism [8]. We report here the presence and chemical identification of two norephedrine oxazolidine derivatives that occur in khat tissues.

Materials and Methods

Plant material and chemical standards

Ten accessions of khat (*Catha edulis*, Forsk.) shrubs were cultivated in open field conditions under commercial growing practices, which include drip irrigation and fertilization, at the Newe Ya'ar Research Center in Northern Israel [8]. These khat plants were six years old at the beginning of this study and originated from seeds collected from a single parent shrub. Additionally, three locally grown cultivars, "Bialik" (Bi), "Lavi" (La), and "Michael" (Mi), were examined. This plant material was made available for research by three families of growers, Bialik, Lavi, and Michael, who employ different growing practices for their plants. The "Bialik" plants were 40 years old, the "Lavi" plants 120 years old, and the "Michael" plants 5 years old. A representative voucher specimen (#Cathaedulis0001) was deposited in the National Herbarium of the Hebrew University of Jerusalem. The plants were identified by Dr. Nativ Dudai. The standards (1*R*)(2*S*)-ephedrine, (1*R*)(2*S*)-norephedrine, and 1-phenyl-1,2-propanedione were purchased from Sigma Chem. Co., while the acetaldehyde standard was purchased from Sigma. Racemic cathinone was synthesized by oxidation of racemic norephedrine with $KMnO_4$ [8].

Extraction of khat samples

For each of the 13 khat accessions, freshly picked young leaves and stems (~ 0.5 g) were crushed to a fine powder with a mortar and pestle under liquid nitrogen. Three milliliters of double distilled H_2O containing 100 µg of (1*R*)(2*S*)-ephedrine as the internal standard (khat does not contain ephedrine) were added to the fine powder, and the sample was shaken for 30 min at 250 RPM before filtering through one layer of Miracloth (Calbiochem) into an 8-mL glass vial. One and a half milliliters of 1 N NaOH were added to the sample to deionize and retrieve the alkaloids in their uncharged form. Methyl *tert*-butyl ether (MTBE), 3 mL, was added to the sample, which was then vortexed for 30 s and centrifuged at 10 g for 5 min to separate the emulsion into two fractions. The top organic fraction containing the alkaloids was collected, and the aqueous residue was reextracted with an additional 3 mL of MTBE. The pooled ether extracts were dried by the addition of anhydrous sodium sulfate and then evaporated under a gentle stream of nitrogen to a final volume of 0.5 mL. One microliter of the solution was then injected into the GC-MS instrument for analysis (see below) [23,24].

GC-MS

The analysis was performed with an Agilent GC-MSD system model 6890 N, interfaced with an Agilent model 5973 N mass spectrometer. The non-chiral Rtx-5 SIL (cross-linked 95% dimethyl – 5% diphenylpolysiloxane) was 30-m long with 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 was set to 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: an initial temperature of 70 °C held for 1 min; and 10 °C/min up to 125 °C for 5.5 min, 2.5 °C/min up to 140 °C for 6 min, and 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, to literature retention indexes, and to the computerized Wiley or PMW TOX2 libraries.

Linearity and detection limit calibration curves were generated using 10 standard dilutions (with 4 replicates) for each of the synthesis products, 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine, at the range of 407–0.4 µg/mL and 335–0.32 µg/mL, respectively. Limit of detection (LOD) values were 0.7 and 0.8 µg/mL for the above two compounds, respectively.

ESI-MS

Electrospray ionization-mass spectrometry (ESI-MS) was performed on a Bruker Esquire 3000 Plus MS instrument. The MS conditions were optimized as follows: API electron spray interface, positive mode polarity, a drying gas flow of 10 L/min, nebulizer gas pressure of 60 psi, drying gas temperature of 335 °C, fragmentor voltage of 0.4 V, capillary voltage of 4451 V, and scan range of *m/z* 25–1000, at 1.15 s/scan. For ESI-MS/MS analysis, the analytical parameters were optimized by infusing the sample solution (1 µg/mL in methanol : water 50 : 50) into the source at a flow rate of 10 µL/min. The optimized parameters were: declustering potential (DP) 200 eV, focusing potential (FP) 400 eV, entrance potential (EP) 12 eV.

Chemical synthesis of 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine

2,4-Dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine were chemically synthesized from norephedrine plus acetaldehyde and *trans*-2-hexenal, respectively, using a 1 : 10 equivalent ratio. Norephedrine was dissolved in 1 : 1 v/v aqueous 1 N NaOH and ethanol solution. The reaction mixture was stirred for 30 min. The NaOH was added to deionize and retrieve the alkaloids in their uncharged form. MTBE, 3 mL, was added to the sample, which was then vortexed for 30 s and centrifuged at 10 *g* for 5 min. The top organic fraction containing the alkaloids was collected, and the aqueous residue was reextracted with an additional 3 mL of MTBE. The pooled ether extracts were dried by the addition of anhydrous sodium sulfate and then evaporated to dryness under reduced pressure. The residue was subjected to silica gel chromatography (eluted with 50% chloroform in methanol) to give the compound. TLC solvent system : methanol: NH_3OH (98 : 0.5 v/v) [25].

^{1}H NMR spectra were recorded on a Bruker DMX-500 instrument operating at 500.1 MHz. Chemical shifts are reported in parts per million (δ), with TMS as the internal standard.

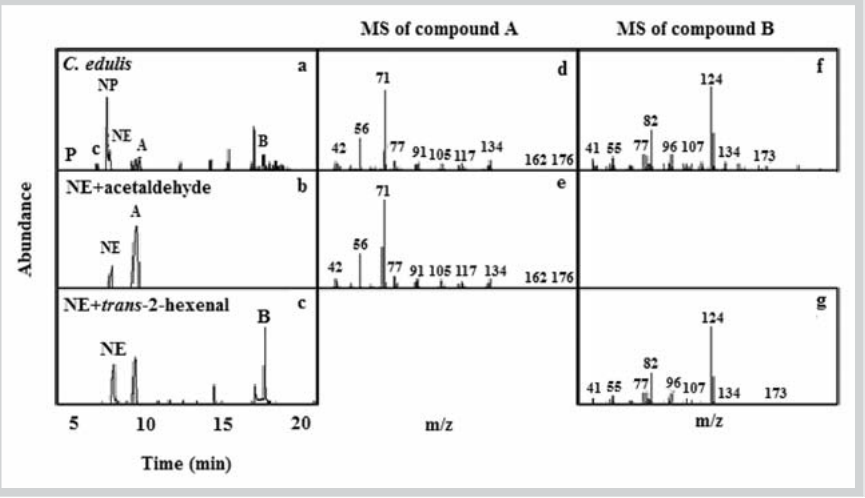

Fig. 1 Mass spectra (GC-MS) of plant extracted and chemically synthesized 2,4-dimethyl-5-phenyloxazolidine (A) and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine (B). The following compounds were also evident on chromatograms: 1-phenylpropane-1,2-dione (P), cathinone (C), norephedrine (NE), and norpseudoephedrine (NP).

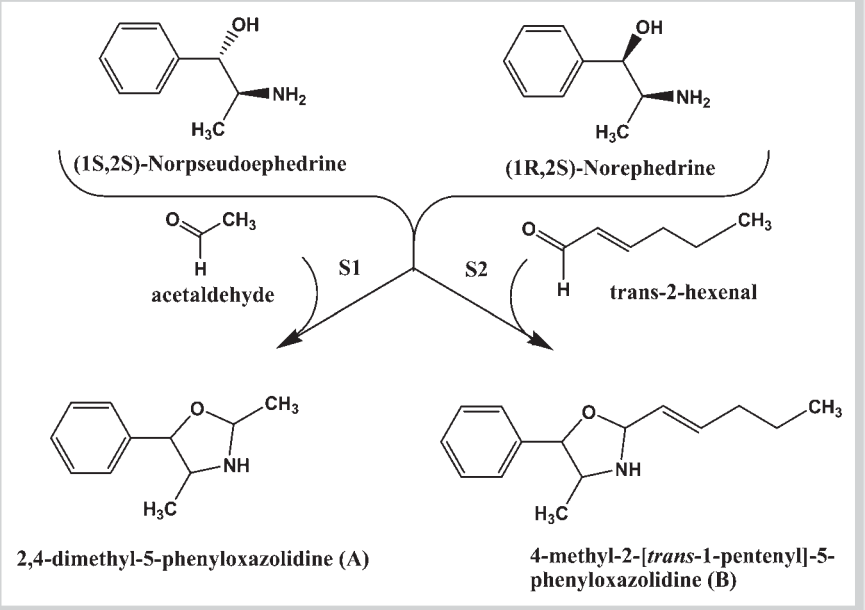

Fig. 2 Synthesis of norephedrine oxazolidine derivatives present in *C. edulis*. Spontaneous conjugations of acetaldehyde to ephedrine alkaloids (reaction S1) forming 2,4-dimethyl-5-phenyloxazolidine (compound A). Spontaneous conjugations of *trans*-2-hexenal to ephedrine alkaloids (reaction S2) forming 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine (compound B).

2,4-dimethyl-5-phenyloxazolidine: ^{1}H-NMR ($CDCl_3$): δ = 7.11–7.22 (m, 5H), 4.97 (d, 1H), 4.62 (d, 1H), 3.56 (m, 1H), 1.25 (d, 3H), 1.10 (d, 3H). ESI-MS (positive mode): *m/z* = 178.1; MS/MS (178.0): 91.0, 115.1, 117.1. Yield ~ 80%; purity ~ 90%.

4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine: ^{1}H-NMR ($CDCl_3$): δ = 7.10–7.20 (m, 5H), 5.67 (m, 2H), 4.93 (d, 1H), 4.61 (d, 1H), 3.54 (m, 1H), 1.98 (m, 2H), 1.40 (m, 2H), 1.20 (d, 3H), 1.05 (m, 3H). ESI-MS (positive mode): *m/z* = 232.2; MS/MS (232.0): 91, 105, 115, 117, 159. Yield ~ 75%; purity 85–90%.

Results

GC-MS analysis of the MTBE extract from khat leaves revealed the phenylpropylamino alkaloids, cathinone, norpseudoephedrine, norephedrine and their metabolic precursor 1-phenylpropane-1,2-dione (**Fig. 1a**). The chromatograms also showed the presence of two additional khat phytochemicals, designated A and B, that could possibly be related to the phenylpropylamino alkaloids. In order to verify the chemical structure of compounds A and B, we chemically conjugated norephedrine and an aldehyde. Compound A was prepared by reacting norephedrine and acetaldehyde to give 2,4-dimethyl-5-phenyloxazolidine (**Figs. 1b** and **2**, compound A). The synthesized compound had an identical retention time and a mass spectrum characteristic to that of the natural product detected in the chromatogram of the khat samples (**Fig. 1d** and **e**). Compound B was synthesized by reacting norephedrine with *trans*-2-hexenal to yield 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine (**Figs. 1c** and **2**, compound B). The synthetic compound B had an identical retention time and a mass spectrum characteristic to that of the natural product detected in khat samples (**Fig. 1f** and **g**). Although norephedrine and acetaldehyde conjugation is a spontaneous reaction, under our extraction conditions this conversion was inadvertent if no aldehyde was added, suggesting that the plant tissues are the source of the conjugates or the aldehydes that conjugate with norephedrine or with norpseudoephedrine present in *C. edulis* tissues, giving rise to compounds A and B (**Fig. 2**).

To verify that 2,4-dimethyl-5-phenyloxazolidine (A) and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine (B) are not artifacts

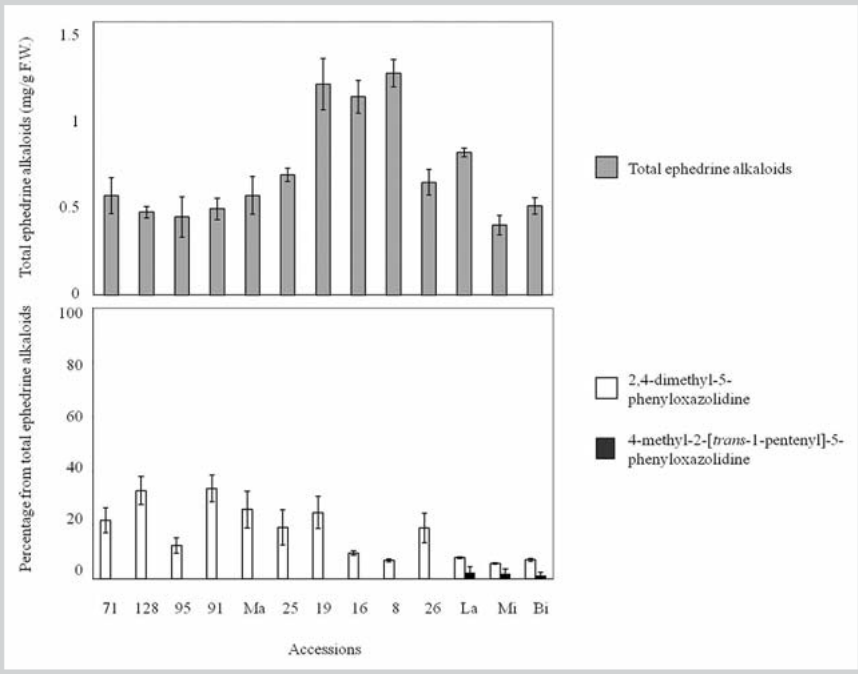

Fig. 3 Norephedrine derivatives in young leaves of different *C. edulis* accessions. Total ephedrine alkaloids (upper panel). Percentage of oxazolidine derivatives from ephedrine alkaloids (lower panel). The results shown are averages of five replicates ± S. E. The numbers in the x-axis represent different Khat accessions, while the letters indicate locally grown cultivars.

unintentionally formed during the extraction of alkaloids from khat tissues, we analyzed, using ESI-MS, plant extracts employing two different extraction procedures. Both alkaline extractions followed by partition with an organic solvent (MTBE) or direct extraction with methanol resulted in the detection of 2,4-dimethyl-5-phenyloxazolidine, with *m/z* 178 $[M + H]^+$, and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine, with *m/z* 232 $[M + H]^+$. We also analyzed, by ESI-MS/MS, the synthesized compounds analogous to the natural compounds and found them to be identical to the natural compounds 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine found in khat tissues. The major ions (*m/z*) 91, 115, 117 corresponding to 2,4-dimethyl-5-phenyloxazolidine as well as ions (*m/z*) 91, 105, 115, 117, 159 corresponding to 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine of the natural compounds were identical to those of the synthetic compounds. In addition, we verified the chemical structure of the synthetic products by ^{1}H-NMR (see Materials and Methods).

To determine the abundance and ratio between phenylpropylamino alkaloids and their oxazolidine derivatives in different khat accessions, leaf extracts from 13 different accessions were analyzed using GC-MS. Marked differences in the concentrations of 2,4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine as a percentage of total phenylpropylamino alkaloids were found (**Fig. 3**). Similarly, all the plant material analyzed contained 2,4-dimethyl-5-phenyloxazolidine at different ratios with respect to total phenylpropylamino alkaloids (calculated as percentage of total phenylpropylamino alkaloids). In some accessions, such as 128 and 91, the levels of 2,4-dimethyl-5-phenyloxazolidine reached 33% of the total phenylpropylamino alkaloids fraction, while in Mi the level was much lower, being as little as 6% (**Fig. 3**). Only three accessions, Li, Mi, and Bi, accumulated low levels of 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine, 2.2, 1.8, and 1.3%, respectively (**Fig. 3**). These data imply that the different khat accessions display a marked polymorphism not only in the amino alkaloid content, but also in the oxazolidine derivatives in a seemingly independent manner.

Discussion

Aldehydes are highly reactive natural plant products that form covalent bonds with available NH_2 and SH groups containing molecules amino acid residues via a Schiff base [27]. We report here the occurrence of two norephedrine oxazolidine derivatives in khat leaves based on identification by GC-MS and ESI-MS/MS and on comparison with their synthetic analogues. Our findings constitute the first evidence for the conjugation of norephedrine and various aldehydes in khat tissues (**Fig. 1**). Under our experimental extraction conditions, norephedrine did not form any oxazolidine derivatives that could be formed in case of aldehyde impurities. Interestingly, 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine was only detected in the homegrown accessions Bialik, Lavi, and Michael and was not present in the accessions under cultivation at the Newe Ya'ar Research Center (numbered accessions). The absence of 4-methyl-2-(*trans*-1-pentenyl)-5-phenyloxazolidine in these accessions could be due to low levels of *trans*-2-hexenal generated in the plant or to different plant chemotypes selected for their psychostimulant effects, cellular or subcellular localization from norephedrine within the plant tissues, or the age of the plants. The data presented here suggests that (1*R*)(2*S*)-norephedrine is not the final product of the phenylpropylamino alkaloids pathway in *C. edulis* as previously thought, but that the metabolic pathway continues downstream to form oxazolidine derivatives. The *N*-methylation step that is prominent in *Ephedra* spp. seemingly does not occur in *C. edulis*. Therefore, it is possible that in this plant most of the downstream flux in the phenylpropylamino alkaloids pathway proceeds through an aldehyde-conjugation mechanism in the form of oxazolidine derivatives. This hypothesis, in turn, suggests that in both *C. edulis* and *E. sinica*, phenylpropylamino alkaloids can serve as precursors for other more complex alkaloid molecules rather

than being the final accumulated products as previously thought. Thus the stabilization mechanism of phenylpropylamino alkaloids in khat might be related to that found in *Ephedra* species. Since *N*-methylation of phenylpropylamino alkaloids does not occur in khat, oxazolidine or oxazolidone formation might be a possible stabilization mode of phenylpropylamino alkaloids in khat tissues. With the advent of better genomic, metabolomic, and transcriptomic platforms to study alkaloid metabolism [26], we will gain much of the needed knowledge to elucidate the biosynthetic pathways to phenylpropylamino alkaloids and their derivatives.

At present, we can only speculate about the ecological roles of phenylpropylamino alkaloids and their oxazolidine derivatives in the plants. It could also be that these compounds may possess interesting and important pharmacological activities. Further research in the unique phytochemistry of khat is needed to better understand the therapeutic potential of this plant.

The ephedrine alkaloids have been subjected to frequent pharmacological examinations, particularly on their actions against the central and autonomic nervous systems. The pharmacological action of the analog of ephedrine, an oxazolidine derivative, was examined mainly on the central and autonomic nervous systems, and contrary to ephedrine, exhibited central depressant actions [28]. It is also shown that a heterocyclic oxazolidone derivative of ephedrine possesses anti-inflammatory activity [16]. We hypothesize that the new oxazolidine we have found in Khat has similar activity to that of ephedrine alkaloid-derived oxazolidine.

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Conflict of Interest

There are no conflicts of interest among authors or the funding agency.

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