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Original Research Article

Mechanistic insight into the cholinergic, muscarinic and antagonistic effects of Khat (*Catha edulis*) on native nicotinic acetylcholine receptors

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Abstract

Purpose: *To investigate the cholinergic, muscarinic and antagonistic effects of Khat (Catha edulis) on native nicotinic acetylcholine receptors (nAChR) and understand its associated mechanism.*
Methods: *Fresh leaves of Catha edulis (50 g) were pulverized and subjected to extraction using 200 mL of methanol and filtered. The filtrate was reconstituted with 0.02 N sulfuric acid, and then chloroform extraction. Properties of the crude alkaloid extract of khat (CAEK) were assessed in skeletal muscles isolated from frog rectus abdominis. In silico analysis of the effects of cathine (CAT) and cathinone (CATO) on muscle-type nicotinic acetylcholine receptors (nAChRs) and molecular docking to predict their potential binding sites on nAChR subunits were carried out.*
Results: *Pre-treatment of isolated muscles with CAEK inhibited carbachol-induced contractility in a dose-dependent manner. At 10, 20, 40 and 80 mg/mL concentrations of CAEK, inhibition percentages were 74.4, 85.2, 95.4 and 99.5 %, respectively. Molecular docking studies show that CAT and CATO modulate the function of nAChRs through competitive antagonism.*
Conclusion: *These results reveal that khat consumption could contribute to the development of skeletal muscle-associated ailments; hence, detailed studies emphasizing cardiac complications and muscular toxicity mechanisms should be conducted.*

Keywords: *Catha edulis, Cathinone, Cathine, Nicotinic acetylcholine receptors, Frog rectus abdominis, Molecular docking*

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INTRODUCTION

Increased chewing of khat (*Catha edulis* (Vahl) Forssk. ex Endl) is associated with many serious health hazards [1]. The effects of khat consumption on the musculoskeletal system such as muscular weakness, skeletal muscle damage, cardiac complications, renal issues, muscle toxicity and possible rhabdomyolysis, have been reported previously. Both CATO and CAT, major alkaloids of khat, have been shown to induce a severe negative inotropic effect on cardiac muscle [2].

Nicotinic acetylcholine receptors (AChRs) play a significant role in neuropharmacology of memory and learning. Therefore, naturally occurring phyto-alkaloids that modulate nAChR activity have garnered attention for improving memory function [3]. Long-term khat consumption in humans is linked to considerable impairments across multiple cognitive domains such as learning, motor speed and coordination, set-shifting and response inhibition, cognitive flexibility, short-term and working memory, and conflict resolution. Rodent studies have repeatedly shown that daily treatment with khat extract results in dose-dependent behavioral deficits, including motor hyperactivity and diminished cognitive function, especially in learning and memory tasks [4]. Despite these negative effects, investigation of naturally occurring alkaloids that modulate nAChR activity continues to be of interest because of their potential to enhance understanding and memory. A previous study revealed that khat contributes to development of muscle toxicity and possible rhabdomyolysis [5].

Therefore, the current study aimed to investigate effect of crude alkaloid extract of khat (CAEK) on nAChRs in isolated frog rectus abdominis, including examining the inhibitory effects of two khat alkaloids, CAT and CATO, on native muscle-type nicotinic AChRs and *Torpedo* acetylcholine receptor (PDB ID: 6UWZ), against acetylcholine and carbamylcholine, and also to predict, by molecular docking, putative sites at which CAT and CATO bind to nAChR subunits.

EXPERIMENTAL

Plant material and extraction

Fresh leaves of *Catha edulis* were supplied by Substance Abuse Research Centre at Jazan University and stored at - 80 °C. The leaves were harvested in accordance with the requirement of the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) organization, with minimal modifications [6]. Thereafter, 50 g of *C. edulis* leaves were processed into fine powder using a mechanical mill. Then, 200 mL of methanol was added and the mixture was sonicated for 15 min. Whatman filter paper No. 1 was used to separate the liquid from solid botanical material and the filtrate was evaporated to dryness using an air stream. A small volume of the solution was reconstituted in 0.02 N sulfuric acid, subjected to chloroform extraction and subsequently separated. The aqueous acidic layer was neutralized with saturated sodium bicarbonate and extracted with dichloromethane. Thin-layer chromatography and liquid chromatography-mass spectrometry were used to assess the activity of this extract for CAT and CATO. The spectroscopic data have been reported in a previous [7].

Ex vivo nicotinic acetylcholine receptors assay

The *rectus abdominis* of frogs were assayed *ex vivo* for nAChR [8]. The frogs were procured from Watania Farm in Khartoum, Sudan. They were first stunned, beheaded and subsequently pithed using a pithing needle. The frogs were placed ventrally on a corkboard and their trunks incised midventrally. Recto muscles were moistened with a frog's ringer solution after the skin was divided along the midline. Two longitudinal slices were taken on each side of the xiphoid cartilage to trace pubis-recti muscle attachment. After cutting the xiphoid cartilage, recti muscles were transferred to a room-temperature ringer solution Petri dish. A thread was placed through each muscle at the top and bottom of the two muscle slices and separated longitudinally along the *Linea alba*. Tissue holders were attached to the bottom threads. The mounted preparation was moved to the organ route, top thread was linked to an isotonic transducer, and a stretching weight was applied to the resting tension to ensure muscle recovery after drug-induced contracture. The physiological salt solution was dissolved in 10 mg/mL carbachol and CAEK before being added to the tissue bath. Control vehicle did not restrict or relax tissue preparation. The study was authorized by the Institutional Ethical Committee of The Medical Research Centre, Jazan University, Saudi Arabia (approval no. MRC/EC/2020/23) and was conducted in accordance with the guidelines on the Care and Use of laboratory animals [9].

Identification and preparation of ligands

Khat alkaloids, CAT and CATO, and two nAChR agonists, acetylcholine (ACh) and carbamylcholine (Figure 1A), were searched using the online database, PubChem (National Center for Biotechnology Information, National Library of Medicine (NLM), Bethesda, MD, USA). Chemical structures of ligands in this study were drawn using ChemSketch, three-dimensional structures of ligands were generated by Open Babel [10] and saved in SDF format for further assessment as well as molecular docking studies.

Identification and preparation of protein target

The structure of Torpedo's acetylcholine receptor (6UWZ), obtained at 2.7-Å resolution from the RCSB PDB (Figure 1B), was subjected to molecular testing using MOE and PyMol software for interactive visualization and analysis. The native muscle-type nAChR and its four subunits underwent geometry optimization, steric clash removal, and charge addition before molecular docking studies. Hydrogen and Gasteiger charges were incorporated while heteroatoms and water molecules were eliminated. Using MOE 2009.10, ten poses for each ligand were docked using default settings, considering interactions and scoring based on London dG and MM/GBVI principles. Best ligand conformers with the lowest binding energy were selected, along with docking scores, binding energy values, and visual representations of docked protein ligands in both 2D and 3D formats [11].

Molecular docking and setup

Molecular docking analyses were conducted using MOE 2009.10, generating ten poses for each ligand while maintaining default values. Superfluous chains were eliminated and ligand interactions were analyzed for cryo-electron proteins to delineate various interaction types as evidence of docking process. Molecular docking was executed using the default parameters of MOE–DOCK, employing London dG scoring algorithm, and retaining 10 ligand conformers with uppermost scores. Scoring arrangement for ligand-target complexes was determined based on energetic criteria (MM/GBVI) and optimal poses with lowest binding energy were chosen for each molecule. The binding energies, docking scores, as well as 2D and 3D chemical configurations of docked protein ligands were subsequently determined.

Statistical analysis

Data was analyzed using the IBM SPSS version 26 and are presented as mean ± standard error of the mean (n = 6). One-way analysis of variance (ANOVA) was used to compare the mean values. The significance threshold was set as 0.05.

RESULTS

Khat compounds interactions with nAChR subunits

Receptor subunits α, β, δ and γ were extracted and their docking results with ligands are shown in Table 1. Cathine (CAT) and cathinone (CATO) showed different binding affinities for all subunits of nAChR.

Antagonistic effects of khat on carbachol-contracted skeletal muscles

Table 2 illustrates the effect of CAEK on carbachol-induced contractile responses in the rectus abdominis muscle. Extract alone did not directly affect contractile responses. However, carbachol-induced contractions were observed to have a significant dose-dependent effect at 1, 5 and 10 ng/mL. Pre-treatment with CAEK led to dose-dependent inhibition of contractile response to carbachol, with inhibition percentages of 74.4 %, 85.2 %, 95.4 %, and 99.5 % at concentrations from 10 to 80 mg/mL, respectively, against carbachol-induced contractions at 10 ng/mL (Table 2).

Docking and molecular interaction

Docking experiments involving CAT and CATO with 6UWZ subunits were compared to the reference drugs, acetylcholine and carbamylcholine. Notably, acetylcholine showed no docking with 6UWZ. On the other hand, CAT and CATO bound to α, β and δ subunits of Torpedo nAChR at sites distinct from acetylcholine and carbamylcholine, except for α-subunit, away from the binding site of a typical agonist (Figure 2, Figure 3 and Figure 4). Cathinone binding to γ subunit coincided with binding sites of acetylcholine and carbamylcholine, indicating potential competitive antagonism of nAChR.

Table 1: Binding affinities of Khat ligands to α, β, δ and γ subunits of nAChR (6UWZ)

Ligand name	Binding affinity (kcal/mol)			
	A	β	δ	γ
Acetylcholine	-	-6.869	-0.805	-7.837
Carbamylcholine	-3.868	-5.370	-0.766	-8.837
CAT	-5.958	-0.982	-5.010	-5.124
CATO	-2.587	-1.562	-0.959	-3.862

Table 2: Antagonistic effects of CAEK on carbachol-contracted skeletal muscles

CAEK (mg/mL)	Contraction inhibition (%)	
	Carbachol (5 ng/mL)	Carbachol (10 ng/mL)
10	79.5±3.10	74.4±5.60
20	91.5±2.30	85.2±2.70
40	98.7±0.90	95.4±1.80
80	100±0.01	99.5±0.90

However, CAT displayed a higher binding affinity than acetylcholine and carbamylcholine for the α and δ subunits (Table 3), suggesting that allosteric inhibition of nAChR could influence the conformation of active site. Molecular interactions of ligands with 6UWZ protein subunits were observed and described, with reference to specific Figures, as follows: carbamylcholine interacted with α-subunit residues including ThrB59, ThrB28, Gln22 and ligand exposure (Figure 2); CAT bound to α-subunit via interactions with Leu B88, ProB 97 and ValB99 (Figure 2B); CATO interacted with α-subunit residues such as ArgB120, ArgB122, AspB49 and GluB98 (Figure 2C). Acetylcholine and carbamylcholine interacted with Gln119, Thr36 and Phe55 of nAChR's β-subunit (Figure 3A and 3B), but CAT's binding is through interactions with Glu182, Ser187 and Ile188 (Figure 3C), while CATO interacts with Val106, and Pro81 of the β-subunit (Figure 3D). Additionally, acetylcholine and carbamylcholine interact with δ subunit residues such as Arg222, Glu191 and Ile193 (Figure 4A and 4B), but CAT interacts with Arg499, Glu175 and Pro173 in δ subunit (Figure 4C), and CATO binds to δ subunit via interactions with Asp171 and Thr164 (Figure 4D). The results of this study show that docking of CATO to γ subunit of 6UWZ involves interactions with five residues similar to those of acetylcholine and carbamylcholine but with direct interactions (Figure 5). Also, CATO exhibited π-π ring stacking interactions with Lys445, a polar side chain acceptor Gln246 and greasy backbone donors including Ala245, Val441, and Pro244 (Table 3), suggesting its potential to competitively antagonize acetylcholine and carbamylcholine. Acetylcholine, on the other hand, interacted with four residues in γ subunit, including basic backbone side-chain acceptors such as Lys445 and Phe244, an acidic side-chain donor, Asp448, as well as a greasy backbone donor, Pro244 (Table 3). Carbamylcholine interactions involved six residues viz three greasy backbone donors (Ala245, Val441, and Pro244), a polar side-chain acceptor Gln246, a basic backbone donor Lys445, and Ser308 (Figure 5B). Furthermore, CATO formed hydrogen bonds with basic backbone acceptors, His27 and Lys23, an acidic side/chain donor Asp30, and a polar side/chain acceptor Asn61 in γ subunit (Figure 5C).

Common residues interacting with CAT and CATO

Table 3 shows the 3D interaction, bond strength and length of residues that formed most communal contact with CAT and CATO. These residues interact with their ligands when their atoms are within four angstroms apart. Similar residues that acetylcholine and carbamylcholine interacted with in columns β and δ are highlighted in black and bold, respectively, as there were no parallel connections with CAT or CATO for the same subunit. However, acetylcholine, carbamylcholine and CATO interacted with similar residues in γ subunit of 6UWZ. Similar residues are highlighted in the same colour.

DISCUSSION

This study investigated the effects of khat on nAChRs using *ex vivo* and molecular docking procedures. An increase in khat use has been linked to several significant health risks. Consumption of khat has also been shown to have a detrimental effect on the musculoskeletal system, resulting in muscular weakening, skeletal muscle damage, cardiac difficulties and potential rhabdomyolysis. Khat alkaloids, CATO and CAT, have been shown to have a significant negative inotropic effect on heart muscle. Furthermore, amphetamine and methamphetamine, which are structurally similar, have been associated with rhabdomyolysis and myoglobinuria [12]. In the present study, pre-treatment with CAEK decreased contractile response to carbachol in a dose-dependent manner. Carbachol is a parasympathomimetic compound that stimulates muscarinic and nicotinic receptors [13]. The findings of this study highlight CAEK's potential to effectively suppress muscle contractility in response to carbachol stimulation, further supporting the effects of khat on muscle-related ailments [5]. This result aligns with previous findings that highlighted the critical role of nAChRs in the neuropharmacology of memory and learning. Naturally occurring alkaloids that modulate nAChR activity have attracted interest because of their potential to modulate memory function [3,14]. However, it is essential to acknowledge that continuing khat chewing in humans is associated with significant cognitive defects across various domains including learning, coordination, motor response inhibition, working memory, cognitive flexibility and conflict resolution.

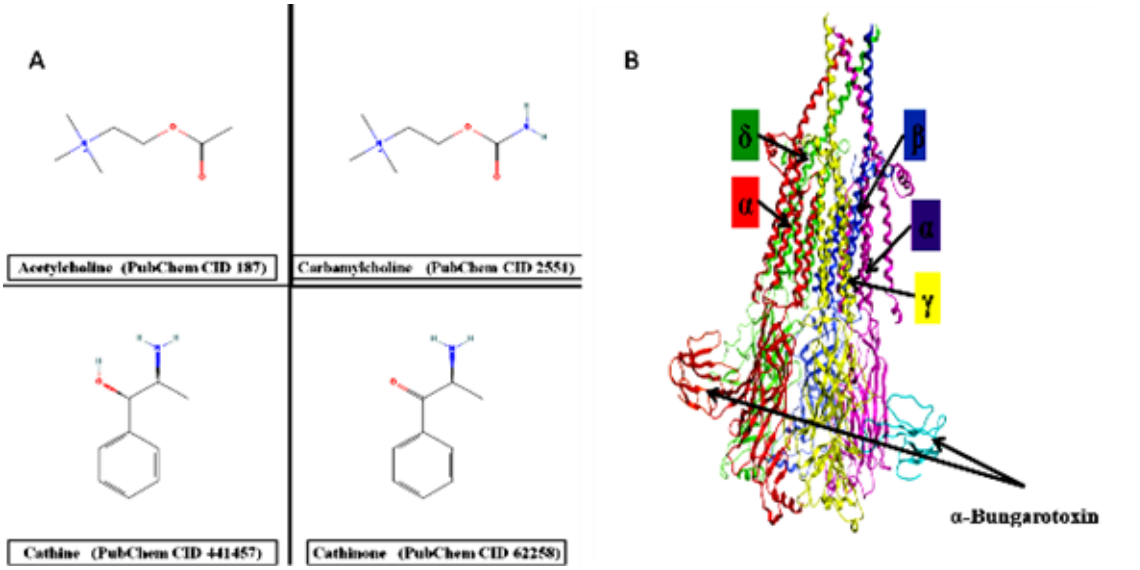

Figure 1: (A) Chemical structures of ACh, carbamylcholine, CAT and CATO. (B) Three-dimensional (3D) structure of *Torpedo* acetylcholine receptors in complex with alpha-bungarotoxin. This AChR-α-bungarotoxin complex exhibited a T-shaped configuration, with homologous sub-units organized in the sequence α-γ-α-δ-β. Two toxin molecules project as handlebars corresponding to the membrane from α-δ and α-γ interfaces in the extracellular domain (ECD).

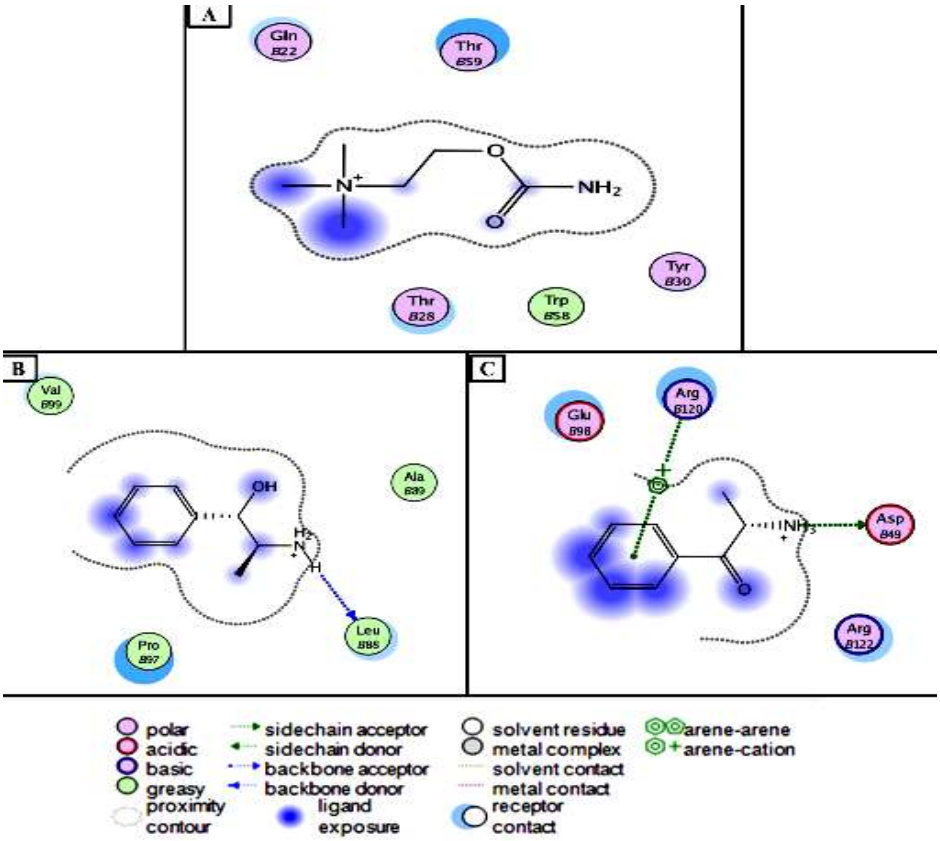

Figure 2: Two-dimensional (2D) view of predicted binding site for carbamylcholine, CAT and CATO to nAChR (A, B, and C) on 6UWZ-α subunit. Green dashed arrows indicate hydrogen bonds, blue arrows indicate backbone acceptor and blue halos indicate solvent-exposed atoms and π-π ring stacking (ArgB120).

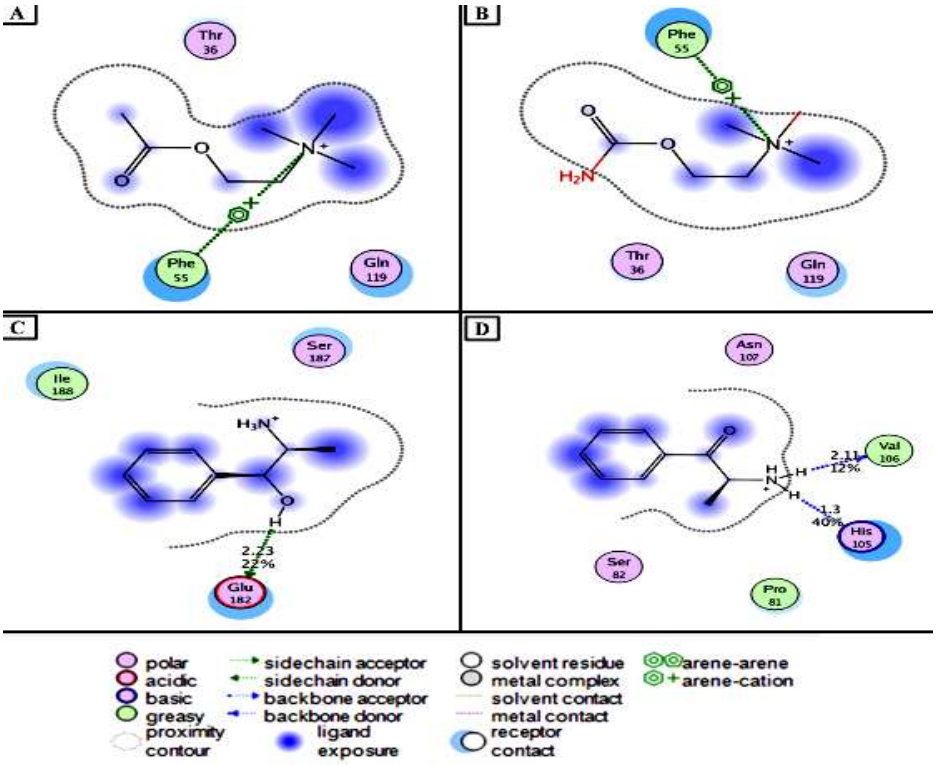

Figure 3: Two-dimensional (2D) view of predicted binding sites for acetylcholine, carbamylcholine, CAT and CATO to nAChR (A, B, C, and D) on 6UWZ-β subunit. Green dashed arrows indicate hydrogen bonds, blue arrows indicate backbone acceptor and blue halos indicate solvent-exposed atoms.

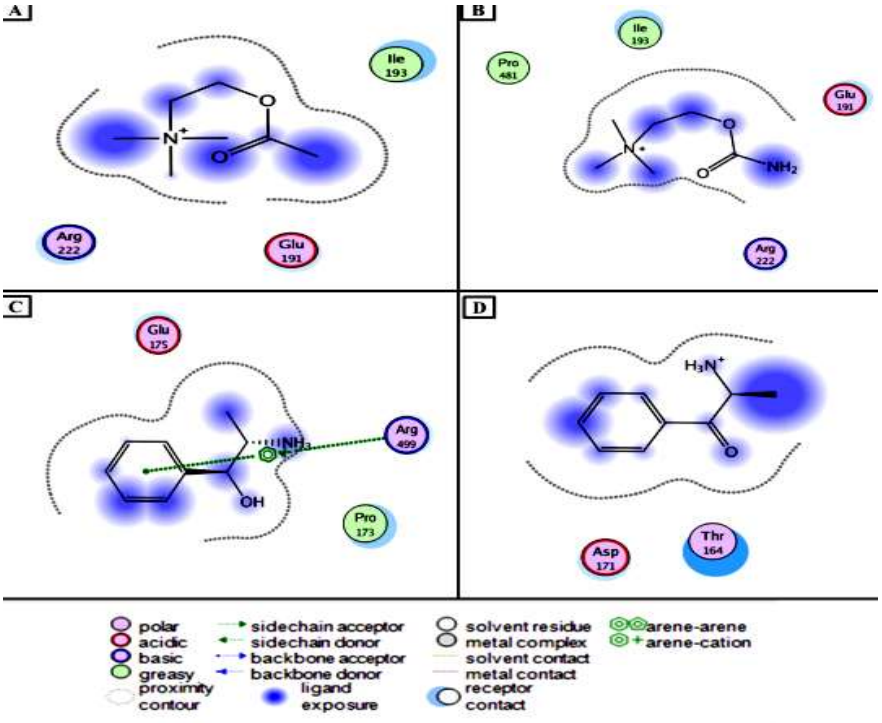

Figure 4: Two-dimensional (2D) view of predicted binding sites for acetylcholine, carbamylcholine, CAT and CATO to nAChR (A, B, C and D) on 6UWZ-δ subunit. Blue halos indicate solvent-exposed atoms. There is π-π ring stacking (Arg 499).

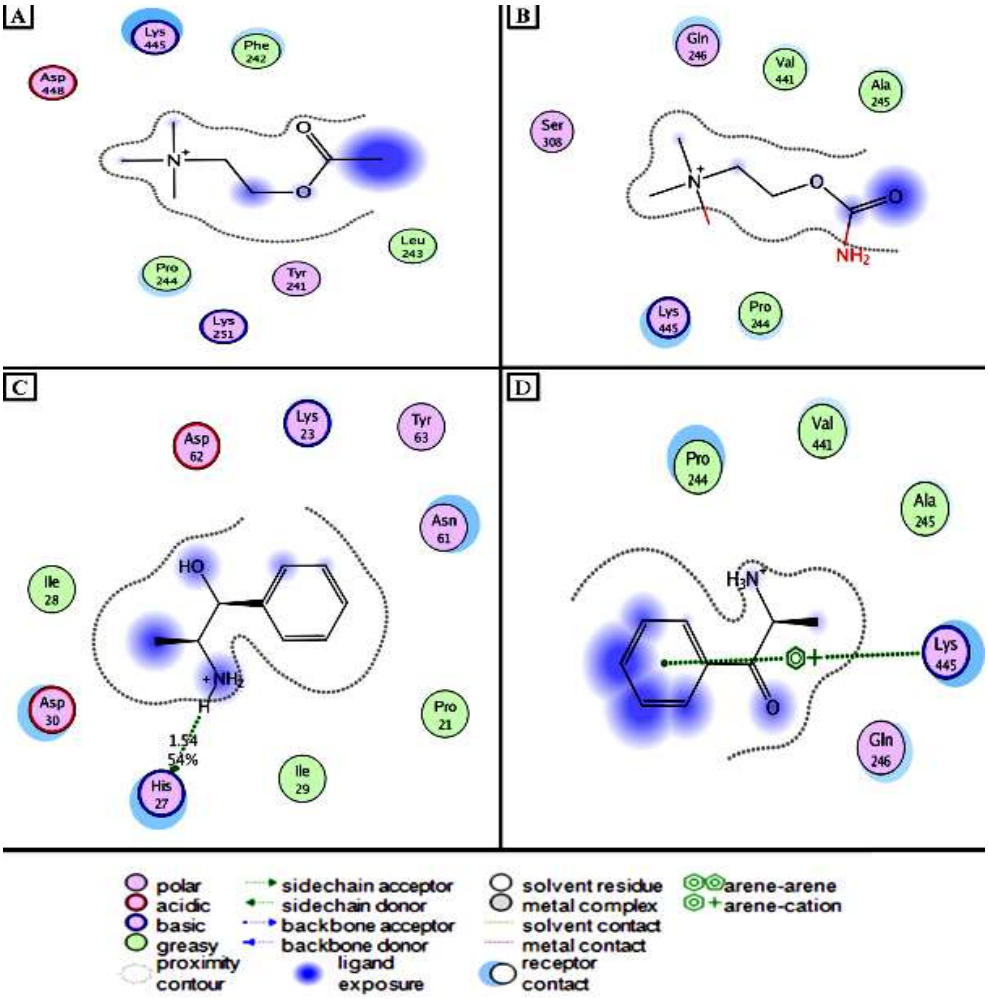

Figure 5: Two-dimensional (2D) view of the predicted binding site for acetylcholine, carbamylcholine, CAT and CATO to nAChR (A, B, C, and D) on 6UWZ-γ subunit. Green dashed arrows indicate hydrogen bonds and blue halos indicate solvent-exposed atoms

Table 3: Common binding residues of selected ligands

Compounds	A	β	δ	γ
Acetylcholine	-	Gln 119	Arg 222	Asp 448
		Phe 55	Glu 191	Lys 445
		Thr 36	Ile 193	Phe 244
				Pro 244
Carbamaylcholine	Thr B59	Gln 119	Arg 222	Ala 245
	Thr B28	Phe 55	Glu191	Gln 246
	Gln 22	Thr 36	Ile 193	Lys 445
			Pro 481	Pro 244
				Ser 308
				Val 441
CAT	Ala B89	Glu 182	Arg 499	Asn 61
	Leu B99	Ile 188	Glu 175	Asp 30
	Pro B97	Ser 187	Pro 173	His 27
	Val B89			Lys 23
CATO	Arg B120	His 105	Asp 171	Ala 245
	Arg B122	Pro 81	Thr 164	Gln 246
	Asp B49	Val 106		Lys 445
	Glu B98			Pro 244
				Val 441

These findings are consistent with rodent studies demonstrating that daily administration of khat extract leads to dose-associated behavioral impairments, such as increased motor activity and reduced cognitive performance, particularly in learning and memory tasks [4]. Despite adverse effects of prolonged khat use, this study underscores the importance of investigating naturally occurring alkaloids that modulate nAChR activity. Understanding the mechanisms underlying these effects may contribute to a better understanding of the memory function.

Also, the result of molecular docking study of nAChR binding site on α, β, δ and γ subunits indicates that the khat compounds, CAT and CATO, are potent modulators of nAChR function. Remarkably, CAT binds to the nAChR with a higher affinity than acetylcholine and carbamylcholine at multiple sites on different subunits, mostly, but not exclusively, to α and δ subunits. The binding of acetylcholine to amino-terminal domains of nAChRs, which then undergo conformational changes that activate the opening of ion channels, is generally believed to be responsible for cholinergic neurotransmission. Enhancing the stability of an allosteric modulator results in an increase in receptor affinity and the likelihood of channel opening [15]. It could be hypothesized that CAT might inhibit nAChR function, probably by an allosteric modulating mechanism or by non-competitive antagonism of acetylcholine.

In this study, α-subunit interactions underscored the importance of positively charged nitrogen in CAT and CATO as a pharmacophoric group, which is inconsistent with a previous study that reported crucial role of nitrogen atoms in this interaction [16]. α-Neurotoxins poison neuromuscular junction by provoking nicotinic receptors on striated muscles through direct competition with acetylcholine, resulting in paralysis, respiratory failure and death [17]. The CATO binds to nAChR with a lower affinity, but exclusively at the same binding site of acetylcholine and carbamylcholine in γ subunit. Current study suggests that CATO may inhibit these functions through competitive antagonism. Moreover, because CATO has been revealed to be the chief active constituent in fresh khat leaves [18], this finding could be a predicted mechanism by which pre-administered khat inhibits carbachol-induced contractility [19].

Limitations of this study

This study utilized frog muscles as a model which could hinder direct applicability of the results to human physiology and also focused on specific effects of khat, neglecting other factors that might also be at play. The study in question deals with cathine and cathinone which does not take into account other phytochemicals contained in khat. Further studies are therefore required to corroborate the findings in this study. Moreover, there are additional implications for regular users of khat that need to be thoroughly studied to increase understanding of the findings beyond muscle activities.

CONCLUSION

Pre-treatment with khat extract decreases contractile response to carbachol in a dose-dependent manner. *In silico* studies reveal an inhibitory effect of khat on musculoskeletal system. There is a need for in-depth studies on cardiac problems, muscle toxicity mechanisms and the phytochemicals involved. Clinicians should be aware of uncommon khat-induced muscular contractions and develop a treatment plan.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. SIA and HMAS orchestrated the investigation, assessed the findings, authored the publications, and

managed the administrative components. AAA and the MMET compiled the facts and amended the language. DAIA helped in responding to the revision in the revised manuscript.

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