Insights into the anorexic mechanism of Khat: an integrated in vivo, ex vivo, and in silico investigations

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Abstract

Background: Chewing Khat (*Catha edulis*) releases cathine and cathinone, which may reduce appetite via an unknown mechanism. This study investigated the peripheral and central effects of fresh leaves and buds of *Catha edulis* water extract (CEWE) on appetite biomarkers, gene expression, and body weight, using in vivo, ex vivo, and in silico models. **Methods:** Rats of both sexes were orally administered CEWE at different doses and durations in three different experiments. Liquid chromatography-mass spectroscopy (LC-MS)-MS was used to detect cathinone and cathine in the murine blood. The effect of Khat on serotonin receptors was studied in isolated rat fundus samples. Docking of the two Khat ligands was performed on G (The 5-hydroxytryptamine-type 2C receptor (5-HT2C) in an agonist-bound active conformation) and H (5-HT2C in an antagonist-bound inactive conformation) proteins to determine which ligands are most likely to act as agonists or antagonists. **Results:** Significant differences ($P < 0.05$) in body weight were observed between the CEWE-treated groups and the controls over eight weeks. However, the plasma leptin and ghrelin levels did not change significantly ($P > 0.05$). The expression of the *ghrelin* and *leptin* genes was also unaffected, but the expression of the *5-hydroxytryptamine* (*5-HT*) gene decreased ($P < 0.05$) with CEWE treatment. CEWE antagonizes 5-HT receptors in isolated rat fundus samples. Docking findings indicated that the khat ligands bound to 5-HT2C receptors. Cathine and cathinone levels in rat plasma were measured. **Conclusion:** Khat extract may suppress appetite by antagonizing the 5-HT receptors. Further research is required to understand its mechanism and potential applications.

Keywords: *Catha edulis*; appetite; leptin; ghrelin; 5-hydroxytryptamine; molecular docking

Highlights

Chewing Khat releases cathine and cathinone, which may reduce appetite via an unknown mechanism. This study investigated the effects of *Catha edulis* water extract (CEWE) on appetite biomarkers, gene expression, and body weight using in vivo, ex vivo, and in silico models. CEWE administration in rats resulted in significant differences in body weight over eight weeks. Plasma leptin and ghrelin levels did not show significant changes with CEWE treatment. The expression of the *5-hydroxytryptamine* (*5-HT*) gene decreased, and CEWE was found to antagonize 5-HT receptors, suggesting its potential role in appetite suppression.

Medical history of objective

In the 14th-century travelogue "*The Travels of Ibn Battuta*", the Islamic scholar and traveler Ibn Battuta documented his journeys throughout the Islamic world, including East Africa. In his account, he mentions the use of Khat by the local people, who would chew its leaves for their stimulating effects.. The use of Khat (*Catha edulis*) as an appetite suppressant has a long-standing history in traditional medicine. Chewing Khat releases cathine and cathinone, which are believed to contribute to its appetite-suppressing effects. While the exact mechanism is not fully understood, studies have shown that Khat can lead to significant differences in body weight over an extended period. However, plasma levels of appetite-regulating hormones like leptin and ghrelin do not appear to be significantly affected. Khat has also been found to antagonize serotonin receptors, specifically the 5-hydroxytryptamine-type 2C receptor (5-HT2C) receptors, suggesting its involvement in modulating appetite through serotonin signaling pathways. Further research is needed to fully comprehend Khat's mechanism of action and potential applications as an appetite suppressant, including comprehensive toxicity evaluations and mechanistic investigations.

Background

Khat (*Catha edulis*) has been widely chewed by the people of East Africa and the Arabian Peninsula for eight centuries [1]. This plant is native to the Hararghe Zone of Ethiopia [2]. The cultivation, marketing, and chewing of Khat are legal in some countries such as Uganda, Ethiopia, Kenya, Yemen, and Djibouti. While Khat is considered a narcotic in Saudi Arabia, its circulation is prohibited in some Western countries [3]. Men and women chew fresh leaves and buds for various purposes. The effects of Khat consumption include increased energy consumption, alertness, and euphoria. Users often experience a sense of well-being, heightened sociability, and desire to engage in conversations. Some individuals also report improved concentration and focus, making it popular among students and workers seeking enhanced productivity [4, 5]. Khat has been incorporated into traditional medicinal practices for various reasons. It has been used to treat conditions, such as depression, headaches, and toothaches [6, 7].

In addition to cathinone and cathine, Khat also contains other psychoactive components, particularly norephedrine, which has similar effects, although to a milder degree [8, 9]. These naturally occurring psychoactive substances are reported to cause excitement, euphoria, increased alertness, and augmented restiveness [7, 10]. According to prevalent traditional beliefs, Khat was reported to reduce appetite, obesity, and body weight [7, 11, 12]. It is well known that amphetamine has appetite-suppressing properties, which may partially explain the low body mass index and malnutrition associated with amphetamine addiction, little is known about their natural equivalents, such as cathine and cathinone [13]. In addition, the action of amphetamine in reducing appetite is well known; it acts through a central mechanism in the arcuate nucleus of the hypothalamus, unlike Khat, which requires further investigation [14].

Dexfenfluramine, structurally similar to amphetamine, is a serotonergic anorectic drug that decreases appetite by increasing extracellular brain serotonin levels [15, 16].

Leptin and ghrelin are two crucial hormones that are involved in the complex regulation of appetite. Leptin, primarily produced by the adipose tissue, acts as a satiety signal, relaying information about the body's energy stores to the brain. High leptin levels indicate sufficient energy reserves, leading to appetite suppression and increased energy expenditure. Leptin acts on receptors in the hypothalamus and other brain regions involved in appetite control, influencing neuronal activity and promoting feelings of fullness [17, 18]. Ghrelin, predominantly secreted by the stomach, plays a pivotal role in stimulating appetite and promoting food intake. Ghrelin levels increase before meals and decrease after eating. It acts on receptors in the hypothalamus, triggering hunger sensations and stimulating food-seeking behaviors. Ghrelin also influences reward pathways in the brain, potentially contributing to the pleasurable aspects of eating [18, 19]. Understanding the interplay between Khat consumption and leptin-ghrelin interactions can contribute to a better understanding of the overall impact of Khat consumption on energy balance and weight management. This may also have implications for potential therapeutic applications or interventions targeting appetite control and weight loss [5, 20, 21].

Previous research has established the anorectic effects of Khat, but the underlying mechanisms remain unclear. While studies have shown that pure cathinone and Khat chewing can suppress appetite, the hormonal pathways involved have yet to be elucidated fully [11, 22, 23]. This study aimed to address this gap by investigating the effects of acute and chronic Khat administration on appetite-regulating hormones (ghrelin and leptin) and serotonin receptors in the gastrointestinal tract (using ex vivo rat tissue). mRNA of these biomarkers was also measured. Additionally, molecular docking simulations were conducted to analyze the interaction between Khat's prominent alkaloids (cathine and cathinone) with 5-HT2C serotonin receptor subtypes. By exploring these aspects, the study seeks to shed light on the mechanisms underlying Khat's anorectic effects.

Materials and methods

Materials and reagents
All solvents, reagents, and chemicals used in this study were analytical grade. Amphetamine, cathine, and cathinone reference standards were obtained from Lipomed (Al-Ashban Trading, Riyadh, Kingdom of KSA). Ethanol, chloroform, sodium chloride, potassium chloride, magnesium sulfate, calcium chloride, glucose, ammonium formate buffer, formic acid, and acetonitrile were purchased from Sigma-Aldrich and Thermo Fisher (St. Louis, MO, USA). Kits for ghrelin (Product Code: MBS2602044) and leptin (Product Code: MBS012834) were purchased from MyBioSource (San Diego, CA, USA).

Plant material and extraction
The Khat plant was obtained from the Substance Abuse and Toxicology Research Center, Jazan University, with official approval. Taxonomic identification was confirmed at the Medical Research Center of Jazan University and a voucher specimen (MRC/CE/1442-1) was deposited. CEWE (Cathe edulis water extract) was obtained using a specific extraction method [10, 24, 25]. Fresh leaves and young shoots of khat plants were frozen, minced, and extracted using distilled water. The resulting filtrate, concentrated by evaporation or freeze-drying, was promptly administered to the animals. This approach aims to preserve the bioactivity and integrity of extracted compounds. The doses were calculated based on fresh plant weight.

Animal study
Animals, husbandry, ethical approval, and sample collections.
Sprague-Dawley rats of both sexes, weighing 150–200 g (n = 70) and eight weeks old, were obtained from the Medical Research Center at Jazan University (Jazan, Saudi Arabia). The rats were disease free and

kept in highly hygienic environment. Ethical permission (approval number: MRC/1443/4/1) was obtained from Jazan University, and animal care followed institutional guidelines of Jazan University Ethical Committee. Rats were acclimatized to specific conditions, housed in polypropylene cages with a 12-h light/dark cycle, and provided free access to food and water. Khat was orally administered using sterile syringes and feeding needles. After diethyl ether anesthesia, blood samples were collected through heart acupuncture or cervical dislocation. Serum and plasma samples were stored at 4 ℃. Animal weights were measured using a digital balance.

Grouping and dosing of experimental animals. In a preliminary experiment, various doses of CEWE ranging from 100 to 2,000 mg/kg were administered to six groups of rats, with no observed signs of toxicity [26]. Doses of 100, 300, and 500 mg/kg were chosen for subsequent studies. Three experiments (Figure 1) were conducted to examine the acute and chronic effects of CEWE. The first study involved both male and female rats, lasting for eight weeks to evaluate long-term anorexic effects. The study involved both male and female rats and lasted for eight weeks to evaluate the long-term anorexic effects of CEWE. The second study focused on acute effects (2 h). It consisted of 20 male rats divided into four groups, including a control group receiving distilled water and three groups receiving oral doses of CEWE at 100, 300, and 500 mg/kg. The third study included five male rats administered a calculated dose equivalent to Khat's average daily human intake (12.4 g), with plasma samples collected two hours after administration for leptin and ghrelin measurements. Collectively, these experiments aimed to investigate the effects of CEWE and Khat on appetite-regulating hormones, providing insights into their anorectic mechanisms. Blood samples were collected for measuring leptin and ghrelin levels. Tissues were promptly frozen for subsequent use in reverse real-time polymerase chain reaction analysis.

Body weight. In the first study (Figure 1), which focused on the long-term effect of CEWE on the weight of rats, live weight was recorded on the first day and the 56th day. The final weights of the animals were determined using a sensitive digital balance. Changes in body weight were assessed using a previously reported method [27]. The initial body weights of all animals were measured before they were orally administered CEWE orally for eight weeks. Weight increase (as a percentage) was computed after the eighth week as follows in Equation (1):

$$\text{Weight Gain \%} = \frac{\text{Final Weight (gm)} - \text{Initial Weight (gm)}}{\text{Final Weight (gm)}} \times 100\% \quad (1)$$

Determination of ghrelin. Ghrelin concentration in plasma was evaluated using a competitive inhibition enzyme immune assay [28]. All reagents and solutions were prepared according to the manufacturer's protocol (BioSource, San Diego, CA, USA). Standards and samples (100 μL) were placed on a microplate in their respective places. Then, 50 μL of Reagent A was added to all wells. A plate sealer was used to cover the microplate. The microplate was then incubated for 1 h at 37 ℃. After incubation, the plate was cleaned three times with wash buffer using a Biotek washer (Model 50TS, Agilent, Jeddah, Saudi Arabia). A 100 μL working solution of detection (Reagent B) was pipetted, and the microplate was covered with a sealer. After 30 min of incubation at 37 ℃, the microplate was washed five times with washing buffer. Then, the substrate (90 μL) was added to each well of the washed microplate, and which was sealed and incubated for 20 min at 37 ℃. The reaction was terminated by adding 50 μL of the stopping reagent. The color change was immediately detected at 450 nm using an ELISA microplate reader (Biotek 800TS, Agilent, Jeddah, Saudi Arabia) equipped with Gen 5 software.

Determination of leptin. Plasma leptin concentration was evaluated using a competitive inhibition enzyme immune assay procured from MyBioSource, San Diego, CA, USA [18]. All the reagents and solutions were prepared according to the manufacturer's instructions. Standards and samples (100 μL) were placed on microplates at their respective positions. The microplate was wrapped with an adhesive plastic film and kept in an oven at 37 ℃ for 120 min. After incubation, 100 μL of the detection solution (Reagent A) was added to all wells, covered with an adhesive plastic film, and kept in an oven at 37 ℃ for 60 min. After incubation, the plate was washed thrice with wash buffer using a Biotek washer (Model 50TS). Reagent B (100 μL of reagent B was pipetted into each well and incubated at 37 ℃ for 60 min. The microplate was then rinsed five times. The substrate was incubated at 37 ℃ for 20 min and then sealed with an adhesive plastic film. Then, a stopping solution was added to block the reaction. An ELISA microplate reader (Biotek 800TS) with Gen 5 software was used to determine the change in color at 450 nm.

Serotonin receptors study

Alkaloids rich extraction. In this experiment, a previously reported method was used to extract Khat, because the goal was to obtain an effective alkaloid concentration, specifically for cathine and cathinone [6]. Briefly, 50 g of green material was macerated into small pieces in a plant mill. After adding 200 mL of methanol, the mixture was sonicated for 15 min. To distinguish the liquid from the solid plant material, the resulting mixture was filtered through a Buchner funnel using a Whatman filter paper. The alcohol solution was evaporated to
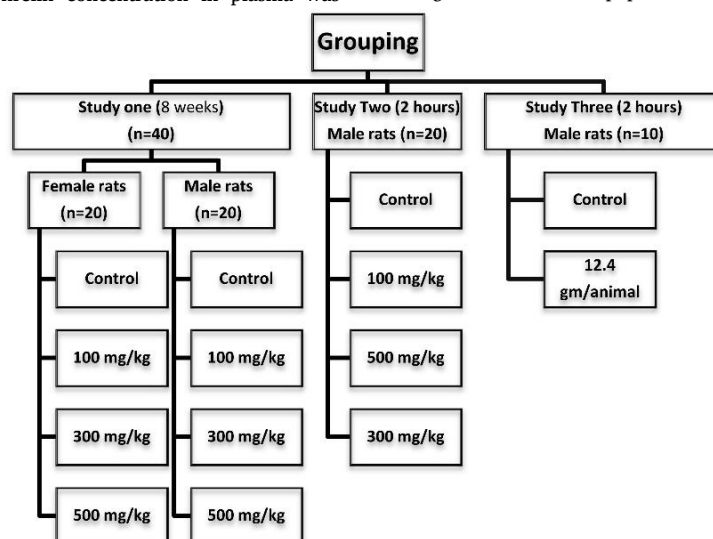
Figure 1 Experimental design of the in vivo study investigating the effects of CEWE on appetite regulation. Three experiments with different durations and doses were conducted to examine the acute and chronic effects of CEWE. The first experiment involved male and female rats (n = 40) and focused on assessing the long-term anorexic effects of CEWE over eight weeks. The rats were administered CEWE orally, and their appetite responses were monitored throughout the study. This experimental design aimed to investigate the potential anorexic effects of CEWE and gather data on its impact on appetite regulation in a long-term setting.

dryness under steam. The small volume was dissolved in 0.02 N sulfuric acid before extraction and separation in chloroform. Saturated sodium bicarbonate was used to make the aqueous acidic layer basic, and dichloromethane was used to remove it.

Rat fundus strip. The experiment was based on a previously reported method [29]. Five rats were used and the experiments were conducted on five different days. The animal was slaughtered by neck dislocation and left for exsanguination; therefore, no anesthetic drugs were used to affect the results. The gastric fundus was removed and placed in fresh Krebs Ringer solution. Two incisions were made on either side of the dome-shaped preparation in order to open the fundal end lengthwise. A strip was created from the tissue by alternating the transverse incisions on the opposing sides of the muscle. Reads were knotted around each end of the strip. One end was linked to a tissue holder and moved to an organ bath; the other was attached to the transducer; a 1-gram stretching weight was placed on the tissue, and it was allowed 45 min to equilibrate. CEWE and 5-hydroxytryptamine (5-HT) were then added to assess their agonist and antagonist effects on the 5-HT receptors. The results are expressed as a percentage of inhibition.

RNA extraction and reverse transcriptase-polymerase chain reaction. In this study, the gene expression of *leptin*, *ghrelin*, and *5-HT* markers was examined using the quantitative reverse transcription polymerase chain (RT-qPCR) technique. RNA extraction from the cells was performed using the TRIzol reagent from Solarbio Life Science, Beijing, China. The amount of extracted RNA was determined using a NanoDrop spectrophotometer. Subsequently, the first strand of cDNA was synthesized using the ABscript II cDNA First-Strand Synthesis kit from AB clonal technologies, Beijing, China. For the RT-qPCR analysis, the $2\times$ universal SYBR green fast qPCR mix, also from AB clonal technologies, Beijing, China, was utilized. The genes of interest, including *leptin*, *ghrelin*, and *5-HT*, were normalized using *β-actin* as the reference gene. The primer sequences for each gene were designed and obtained from Macrogen, Seoul, Korea. The relative expression levels of these genes were calculated using the $2^{-\Delta\Delta Ct}$ method [30]. The primer sequences used for PCR amplification were as follows:

Murine β-Actin:
Forward: AAGGCCAACCGTGAAAAGAT
Reverse: GTGGTACGACCAGAGGCATAC
Ghrelin:
Forward: AAGTAACCACTGACAGGACTGGT
Reverse: CAGGAGAGTGCTGGGAGTTGCA
Leptin:
Forward: TGCTGCAGATAGCCAATGAC
Reverse: GAGTAGAGTGAGGCTTCCAGGA
5-HT:
Forward: CTCCTCCCGATCTCTTTGAAATCGC
Reverse: TGTTCGAGCTTTGCCCAGTTCG

Liquid chromatography-mass spectrometry
Calibration and control samples were prepared by dissolving pure standards of cathinone, cathine, and amphetamine in a negative plasma sample. Reference samples of d,l-cathinone HCl and d-cathine HCl were also prepared. The concentrations used for calibration curve establishment ranged from 20 to 1,000 ng/mL. Solid-phase extraction was employed to extract one mL of blood, calibrators, and control samples, followed by drying under a nitrogen stream and reconstitution in a 150 mL mobile phase [31]. Liquid chromatography-mass spectroscopy (LC-MS)-MS analysis was conducted using an LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher, Jeddah, Saudi Arabia) attached to an autosampler. Liquid chromatography analysis was performed on a C18 column using an isocratic technique with a mobile phase consisting of ammonium formate buffer. The flow rate was set at 300 mL/min. The compounds were detected using electrospray ionization, and the mass spectra were obtained in the full scan mode. Cathine, cathinone, and the internal standard amphetamine-D5 were identified and quantified using collision-induced dissociation of precursor ions [32].

Molecular docking
Ligands' identification and preparation. The Khat-associated ligands cathine and cathinone and available drugs for weight control, obtusifoliol, and cassiaside B2 (Supplementary Figure S1), were obtained from PubChem (National Center for Biotechnology Information, National Library of Medicine, USA), an online database [33]. ChemSketch was used to create the chemical structures of the ligands [34]. Open Babel software was used to produce three-dimensional structures of the ligands, which were then saved as Single-User Geodatabase file format for further preparation and molecular docking studies [35].

Identification and preparation of protein targets. The protein structures of serotonin receptors (5-HT2C) are shown in Supplementary Figure S2. Their structures were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) [36]. Two 5-HT2C serotonin subtype receptors were chosen as target proteins for the docking investigations, as shown in Supplementary Table S1. These were later referred to as "G" (5-HT2C in an agonist-bound active conformation; chain-A of PDB ID: 6BQG) and "H" (5-HT2C in an antagonist-bound inactive conformation; chain A of PDB ID: 6BQH), respectively. To unveil the structural conformation of G and H, the two proteins' amino-acid residues were superimposed using sequence alignments of residues on Pymol. All extracellular loops are well resolved in both 5-HT2C crystal structures. Superposition of the G and H complexes shows pumps, indicating that they represent different conformational states of the receptor (Supplementary Figure S3). Molecular Operating Environment (MOE) and PyMol software were used to visualize and analyze molecular structures in an interactive manner so that protein-ligand interactions, geometry optimization, and the pre-docking technique could be better understood. Hydrogens and Gasteiger charges were added to the protein structure, and all heteroatoms and water molecules were removed. MMFF94's force field parameters were used to optimize protonated proteins to eliminate unfavorable steric conflicts [37, 38]. The protonated and optimized structures were preserved for further preparation and analysis. MOE software was used to execute all minimizations (grade < 0.001).

Molecular docking and setting. MOE was used to conduct docking tests with ten poses for each ligand (the other parameters were kept at default). Superfluous chains were removed. Different types of ligand interactions with the X-ray co-crystallized protein were determined, so that the next docking technique could be used. Docking was accomplished using MOE's default DOCK settings, which were as follows: the scoring function was London dG., and the ten ligand conformers with the highest and best scores were preserved by default. The scoring configuration of the ligand-target complexes was determined based on energetic grounds (MM/GBVI). For each compound, the optimal positions with the lowest binding energies were chosen. Docking scores, binding energies, and chemical structures of the selected ligands are shown.

Toxicity and drug likeness prediction. A web server called ProTox was used to predict the acute oral toxicity of cathine and cathinone based on their chemical similarities with known toxic properties and presence of toxic fragments [39]. Toxicity classifications were defined using the admetSAR program, in line with the worldwide standardized system of chemical categorization and labeling [33]. Classes I and II are fatal, class III is toxic, class IV is harmful, class V may be harmful, and class VI is non-toxic in nature. Toxic doses were specified as lethal dose 50% (LD_{50}) values in mg/kg body weight, where LD_{50} is an approach that yields an in silico drug compound for particular proteins. The pharmacokinetics and drug-likeness of the ligands were evaluated using SwissADME [40]. SwissADME is a web-based tool for this purpose.

Statistical analysis
The data were analyzed using SPSS version 21.00. The data are presented as mean $\pm$ S.E.M. One-way analysis of variance (ANOVA) and Student's t-test were used to compare the mean differences. A full factorial ANOVA was used to analyze the body weight based on sex

and group. The L.S.D. test was used for post-hoc analysis. Alpha was set at 0.05.

Results

Effects of CEWE on weight gain
A full factorial ANOVA was conducted to analyze the effects of sex and dose on weight-related measurements (Table 1). The results indicated that both sex and dose had significant effects ($P < 0.05$). Female rats had a significantly lower final weight (240.6 ± 16.1 g) compared to male rats (295.2 ± 38.4 g) ($P < 0.05$). Additionally, there was a significant change in weight gain percentage between the CEWE-administered group (100 mg/kg) and the control group ($P < 0.05$). No significant interaction between sex and group was observed ($P > 0.05$) in the model.

Levels of leptin and ghrelin
The effects of eight weeks of oral CEWE dosing on plasma leptin and ghrelin levels in male rats were measured using enzyme-linked immunosorbent assay (ELISA). Leptin levels in the normal control groups at doses of 100 mg/kg, 300 mg/kg, and 500 mg/kg showed no significant differences ($P > 0.05$), with mean values of 0.58 ± 0.18 ng/mL, 0.30 ± 0.15 ng/mL, 0.76 ± 0.30 ng/mL, and 0.76 ± 0.33 ng/mL, respectively (Figure 2). Plasma ghrelin levels were not significantly altered by CEWE administration in any of the experimental groups at the end of the study (Figure 2). The 2-h oral administration of CEWE at doses of 100 mg/kg, 300 mg/kg, and 500 mg/kg (Figures 2A, 2B) and a high dose equivalent to the daily human

Table 1 The effects of Khat extract administration on male and female rats' body weight. The duration of the experiment was eight weeks (First study)

Dose (mg/kg body weight/day)	Sex of the rats				Total for both sexes	
	Female		Male			
	Final weights (gm)	Weight gain (%)	Final weights (gm)	Weight gain (%)	Final weights (gm)	Weight gain (%)
Control groups	246.4 ± 12.1[a]	18.26 ± 1.9[a]	299.0 ± 42.4[a]	50.60 ± 7.5[a]	269.77 ± 8.95[a]	32.63 ± 1.2[a]
100 mg/kg	233.8 ± 10.4[a]	14.79 ± 2.6[a]	270.6 ± 9.9[a]	19.23 ± 2.5[b]	252.20 ± 11.63[a]	17.01 ± 0.9[b]
300 mg/kg	232.6 ± 17.1[a]	14.14 ± 1.5[a]	287.4 ± 18.4[a]	24.55 ± 3.6[b]	260.00 ± 3.38[b]	19.34 ± 0.8[c]
500 mg/kg	251.8 ± 20.3[a]	18.86 ± 0.9[a]	324.4 ± 14.2[a]	38.67 ± 5.8[c]	292.11 ± 5.58[c]	29.87 ± 2.5[a]
Total	240.6 ± 16.1[a]	16.39 ± 0.8[a]	295.2 ± 38.4[a]	32.35 ± 1.5[c]	267.86 ± 10.10[d]	24.37 ± 1.8[a]

Groups with different superscript letters are significantly different ($P < 0.05$). SPSS software was used to analyze the data. ANOVA was used to test the differences between the groups' means, followed by post hoc analyses.
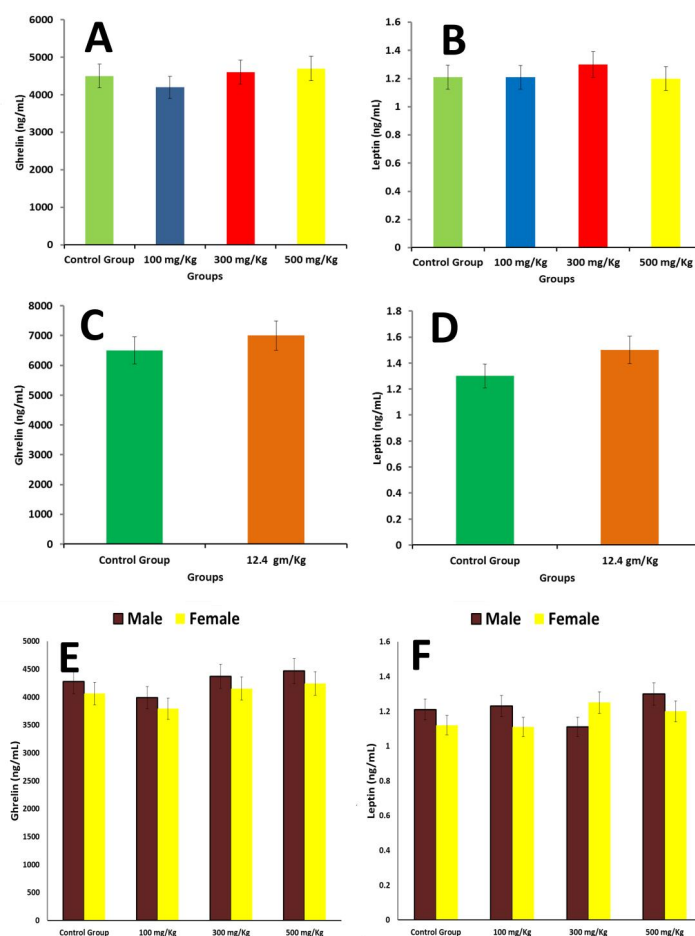
Figure 2 Effects of CEWE ghrelin and leptin. The effects of a 2-h oral administration of CEWE (100, 300, and 500 mg/kg body weight) on the plasma levels of ghrelin (A) and leptin (B). Figures 2C, 2D display the effects of a 2-h oral dose of CEWE (12.4 gm/kg) on the plasma levels of ghrelin and leptin, respectively. Hormone levels in male rats were measured using an ELISA kit. Figures 2E, 2F show the findings for eight weeks. Values are presented as mean ± standard deviation (n = 5). ANOVA and SPSS were used to examine differences ($P < 0.05$) between the different groups. Leptin and ghrelin levels were measured in ng/mL.

dose of CEWE (12.4 gm/kg) (Figures 2C, 2D) did not result in any changes in leptin and ghrelin levels. The findings of the eight-week experiment were presented in Figure 2E, 2F.

Antagonistic effect of Khat extract on 5-HT receptors

In Figure 3, the concentration-response curve of rat fundus strips (n = 5) in the presence of different concentrations of 5-HT was depicted. The highest contraction of gastric tissue was observed at a concentration of 4 ng/mL, and the response exhibited a concentration-dependent relationship ($P < 0.05$). Importantly, pre-administration of Khat extract at a concentration of 50 μg/mL completely antagonized the effects of 5-HT at a concentration of 1 ng/mL. This indicated that Khat inhibited the contractile effect of 5-HT on rat fundus tissue in a dose-dependent manner, as illustrated in Figure 3. Nitrendipine was utilized as a control to inhibit the contractile effects of 5-HT.
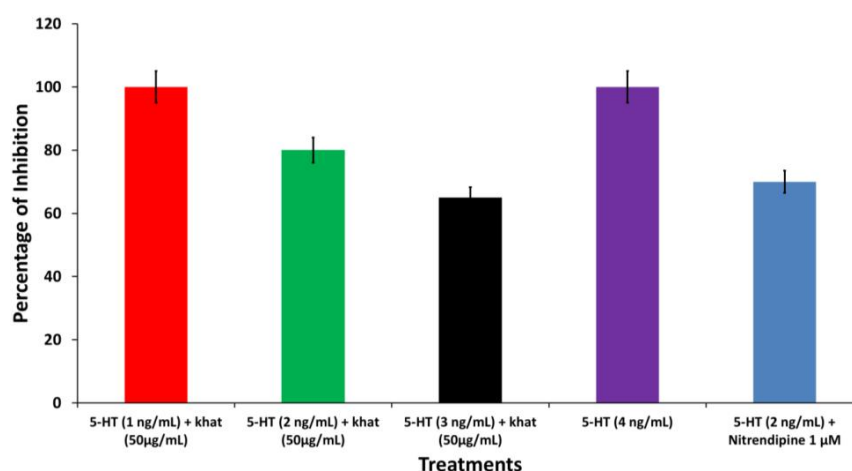
Gene expression

Figure 4 displayed the effects of 2 h's oral administration of CEWE at different doses (100 mg/kg, 300 mg/kg, and 500 mg/kg body weight) on gene expression levels of *ghrelin*, *leptin*, and *5-HT*. In the control group, the expression of *leptin* mRNA was measured as 1 ± 0.01-fold of the control. Following treatment with CEWE at the tested doses, the fold control values for *leptin* expression were 1.1 ± 0.01, 1.12 ± 0.009, and 1.1 ± 0.008, respectively. These results indicated that no significant changes were observed in the expression of *leptin* in response to CEWE treatment at any of the tested doses. Similarly, no significant differences were observed in *ghrelin* mRNA levels among the groups (Figure 4). On the other hand, the gene expression levels of *5-HT* were analyzed, and treatment with CEWE at doses of 100 mg/kg, 300 mg/kg, and 500 mg/kg resulted in a significant decrease in *5-HT* expression compared to the control group ($P < 0.05$). The decrease in *5-HT* expression was dose-dependent, with the highest dose of 500 mg/kg showing the most pronounced reduction.

Figure 3 The experimental setup for assessing the effects of 5-HT and Khat on rat fundus strips. The experiments were conducted using five rats on separate days. The gastric fundus was isolated and placed in Kreb's ringer solution. Khat extract was added to investigate its agonist and antagonist effects on 5-HT receptors. Notably, the preadministration of Khat extract at 50 μg/mL completely antagonized the effects of 5-HT at a concentration of 2 ng/mL. Additionally, Khat inhibited enzyme activity by 51% at a concentration of 50 μg/mL. 5-HT, 5-hydroxytryptamine.
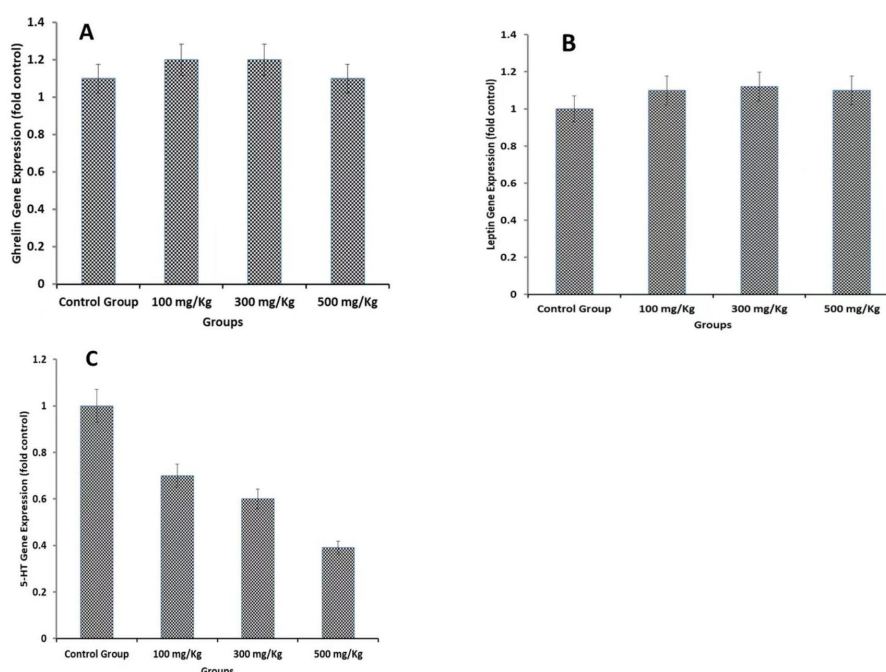

Figure 4 The relative expression levels of *ghrelin* (A), *leptin* (B), and *5-HT* (C) were analyzed using RT-qPCR. No significant effect was observed in the expression of both *ghrelin* and *leptin* genes. However, treatment with CEWE resulted in a decrease in the expression of the *5-HT* gene. The data represents the mean ± standard deviation (n = 5). Statistical significance was determined using a one-way ANOVA test.

LC-MS-MS quantification of Khat components in plasma
In Figure 5, cathine and cathinone were observed to have well-defined peaks with retention times of 6.02 and 6.09 min, respectively. LC-MS-MS in full scan mode provided high sensitivity for detecting these Khat alkaloids. There were no significant endogenous peaks detected in the chromatograms of blank plasma samples that interfered with the cathine or cathinone peaks. The calibration curve, ranging from 20 to 1,000 ng/mL, exhibited excellent linearity with a Pearson's correlation coefficient of 0.9998. In the plasma samples of rats treated with 300 mg/kg Khat extract, cathine and cathinone were identified. The concentrations of cathine and cathinone were determined to be 28.72 ng/mL and 91.13 ng/mL, respectively.

Molecular docking and in silico prediction of toxicity and pharmacokinetics
Khat compound interactions with 5-HT2C: putative agonists and antagonists. Docking of the two Khat ligands was performed on G (5-HT2C in an agonist-bound active conformation) and H (5-HT2C in an antagonist-bound inactive conformation) proteins to determine which ligands are most likely to act as agonists or antagonists. Figures 6, 7 show how well cathine and cathinone docked to the G and H 5-HT2C proteins compared to how well the reference drugs bound to those proteins. Both cathine and cathinone bound to G, but at different sites for cassiaside B2 and obtusifoliol, away from the hydrophobic pocket that resembles the agonist-binding site (Figures 6A, 6B). However, cathine and cathinone had relatively higher binding affinities than that of obtusifoliol (Figures 6C, 6D). Figures 6C, 6D show that cathine and cathinone bound to H at the same binding sites as those of the reference compounds (Figures 7A, 7B). As a result, we examined the relative differences in the binding affinities of G and H to classify the Khat ligands as acceptable agonists and antagonists. Inspection of the contacts between cathine and G protein indicated two residues with a combination of polar, non-polar, and acidic residues (Figures 7A, 7B). The two reference compounds exhibited positive free energy (ΔΔG) values greater than +1 kcal/mol (cassiaside B2) with ΔΔG = +1 kcal/mol (obtusifoliol) and ΔΔG = +0.6 kcal/mol. These results confirmed the agonistic activity of the reference compounds. However, cathine and cathinone exhibited negative ΔΔG; cathine, with ΔΔG = −1.6, and cathine and cathinone, with ΔΔG = −0.3 (Table 2). These results indicated that the Khat ligands had antagonistic activity against 5-HT2C.
Proposed agonist and antagonist ligand protein interactions. In addition to the free binding energy, the molecular interaction between the ligands and the binding site of the two 5-HT2C serotonin receptors is essential for determining whether the ligands can be recognized as agonists or antagonists. First, molecular interactions were observed between cassiaside B2 and obtusifoliol, the standard ligand, and two 5-HT2C serotonin receptors. As shown in Figure 8A, cassiaside B2 is

Table 2 Binding affinity and difference in ΔΔG values between the predicted binding affinity of each Khat ligands to G and H

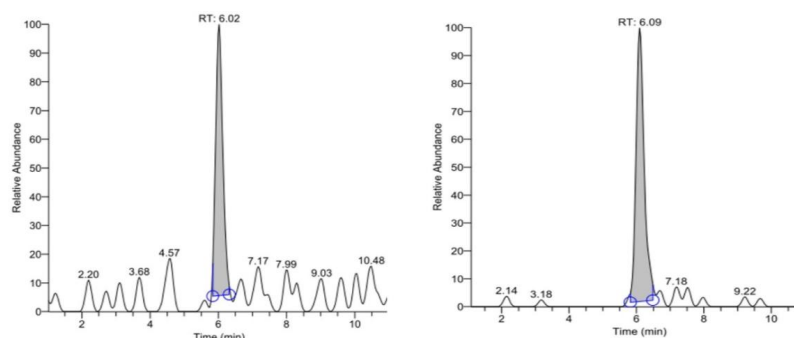| Ligand name	Formula	MW/g/mol	Binding affinity (kcal/mol)		ΔΔG (kcal/mol)
			G	H	(H-G)
Cassiaside B2	$C_{39}H_{52}O_{25}$	920.82	−10.2	−9.2	1
Obtusifoliol	$C_{30}H_{50}O$	426.72	−6.5	−5.9	0.6
Cathine	$C_9H_{13}NO$	151.21	−6.8	−8.4	−1.6
Cathinone	$C_9H_{11}NO$	149.19	−7.5	−7.8	−0.3

Figure 5 Liquid chromatography-mass spectrometry-mass spectrometry of cathine (left image) and cathinone (right image) alkaloids in rat plasma after administration of 300 mg/kg of Khat extract. Mass spectrometry analysis of diluted (1:1,000) STD cathine (a: Ion m/z 152) and STD cathinone (b: Ion m/z 150) shows specific ion scan spectra.
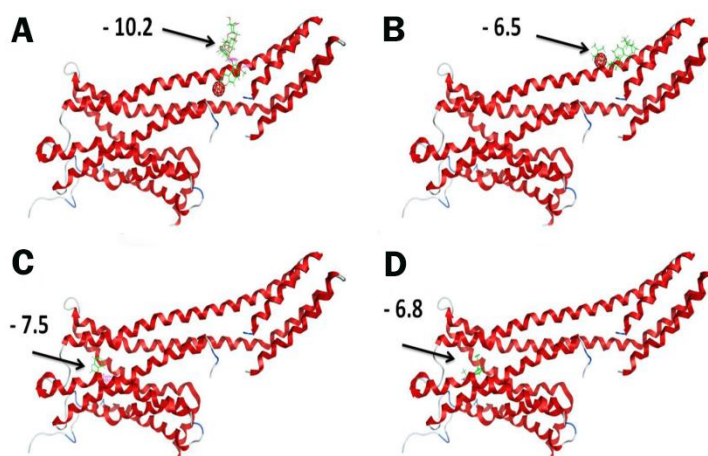

Figure 6 Binding of cassiaside B2, obtusifoliol, cathine, and cathinone (A, B, C, and D, respectively) to structure G. 5-HT2C in an agonist-bound active conformation; chain A of PDBID: 6BQG.
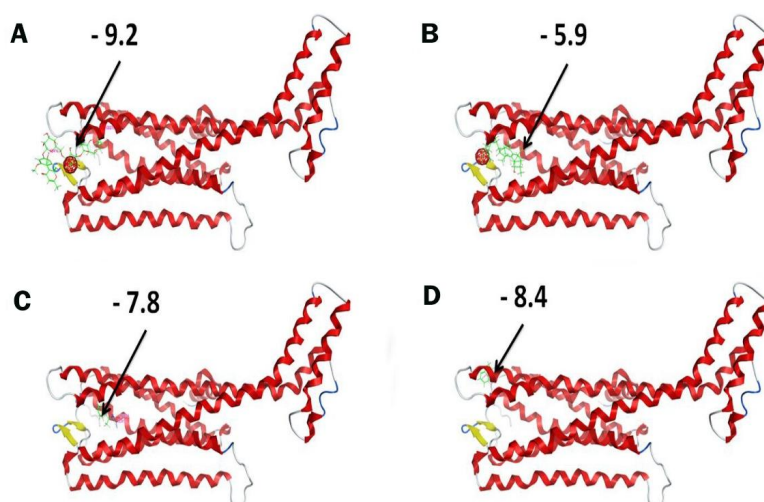
Figure 7 Binding of cassiaside B2, obtusifoliol, cathine, and cathinone (A, B, C, and D, respectively) to structure H. 5-HT2C in an antagonist-bound inactive conformation; chain A of PDBID: 6BQH.

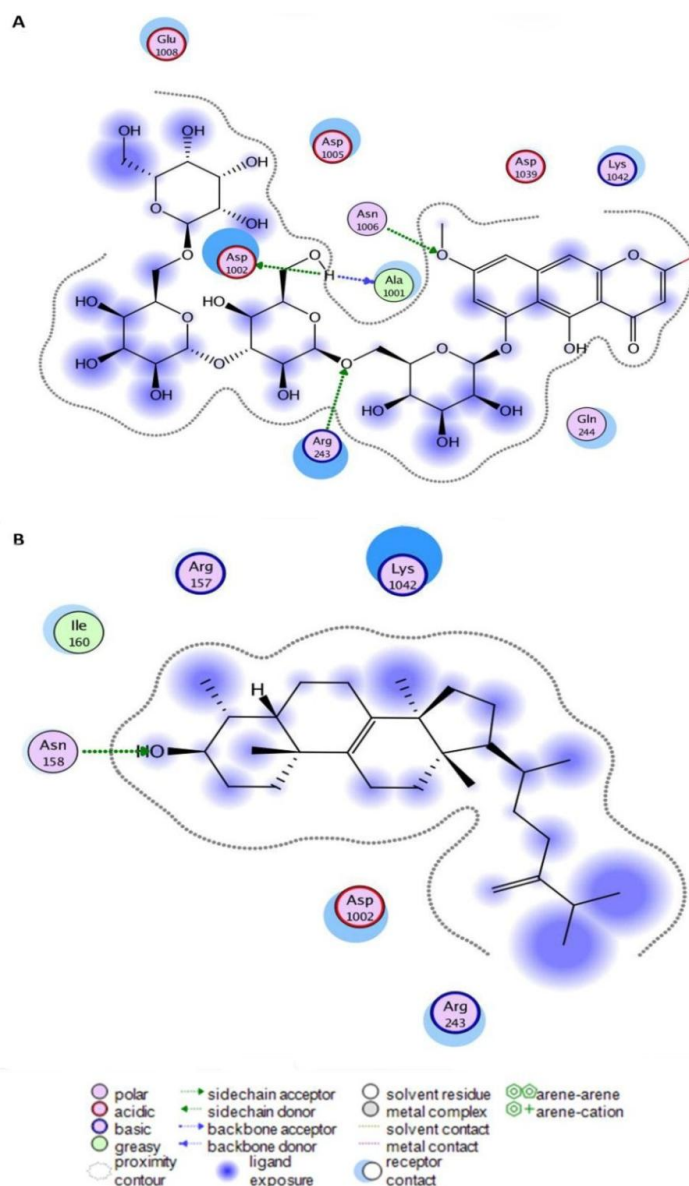**Figure 8 Cassiaside B2 and obtusifoliol interactions at the G binding site.** The 2D ligand-protein interactions of cassiaside B2 and obtusifoliol in the binding site of G (A and B) are indicated by green dashed arrows, indicating hydrogen bonds. Blue halos indicate solvent-exposed atoms.

shown to interact with the amino acids of the G protein as follows: Asp 1002, Arg 243, Asn 1006, and Ala 1001 via hydrogen bonds. Moreover, cassiaside B2 exhibits nonpolar interactions with Asp 1005, Asp 1039, Glu 1008, and Lys 1042, in addition to the polar side-chain acceptor Gln 244. In contrast, the molecular interaction of obtusifoliol with the G protein, as presented in Figure 8B, displayed interactions with six amino acids via hydrogen bonds with the polar side chain acceptor Asn 158. In addition, there is a nonpolar acidic side-chain donor with Asp 1002 and a backbone of Arg 157, Arg 243, Lys 1042, and Ile 160. An inspection of the contacts for cathine with G protein, as shown in Figure 9A, indicates that four residues interact via hydrogen bonding with a non-polar acidic side chain donor with Asp 134. In addition, cathine interacted with the backbone of Val 135 and phenylalanine residues, including Phe 327 and Phe 328. Similarly, inspection of cathinone contacts with the G protein, as shown in Figure 9B, revealed seven combinations of non-polar and polar residues. Interestingly, cathinone displays π-π ring stacking interactions with Phe 327, polar side chains with Tyr 358, and non-polar interactions with Asp 134 and the backbone of Val 135, Val 354, Ile 131, and Trp 324.

Similarly, the molecular interactions between the ligands and binding site of the H protein were examined to determine whether cathine and cathinone could be recognized as antagonists. First, the molecular interaction between cassiaside B2 and obtusifoliol, the standard ligand, and H protein was observed. As shown in Figure 10A, cassiaside B2 interacts with 14 amino acids of the H protein, forming hydrogen bonds with the polar side chain acceptor Asn 203 and backbone donor Leu 350. There are also contacts with non-polar interactions between the acidic side chain donors Glu 198 and Glu 347, backbone donors Leu 209, Val 354, Val 202, and Val 208, and two residues of phenylalanine, including Phe 201 and Phe 327. Moreover, cassiaside B2 was in contact with the polar side-chain acceptors Asn 210, Asn 351, Gln 343, and Tyr 118. In contrast, the molecular interaction of obtusifoliol with the H protein, as presented in Figure 10B, displayed interactions with 12 amino acids and the polar side chain acceptors Asn 331 and Asn 210. In addition, there is a non-polar acidic side-chain donor with Asp 134 and Glu 347. In addition, there were interactions with the backbone donors Ile 131, Leu 209, Leu 350, Trp 130, Val 202, Val 208, Val 354, and Phe 327. An inspection of the contacts for cathine with the H protein, as shown
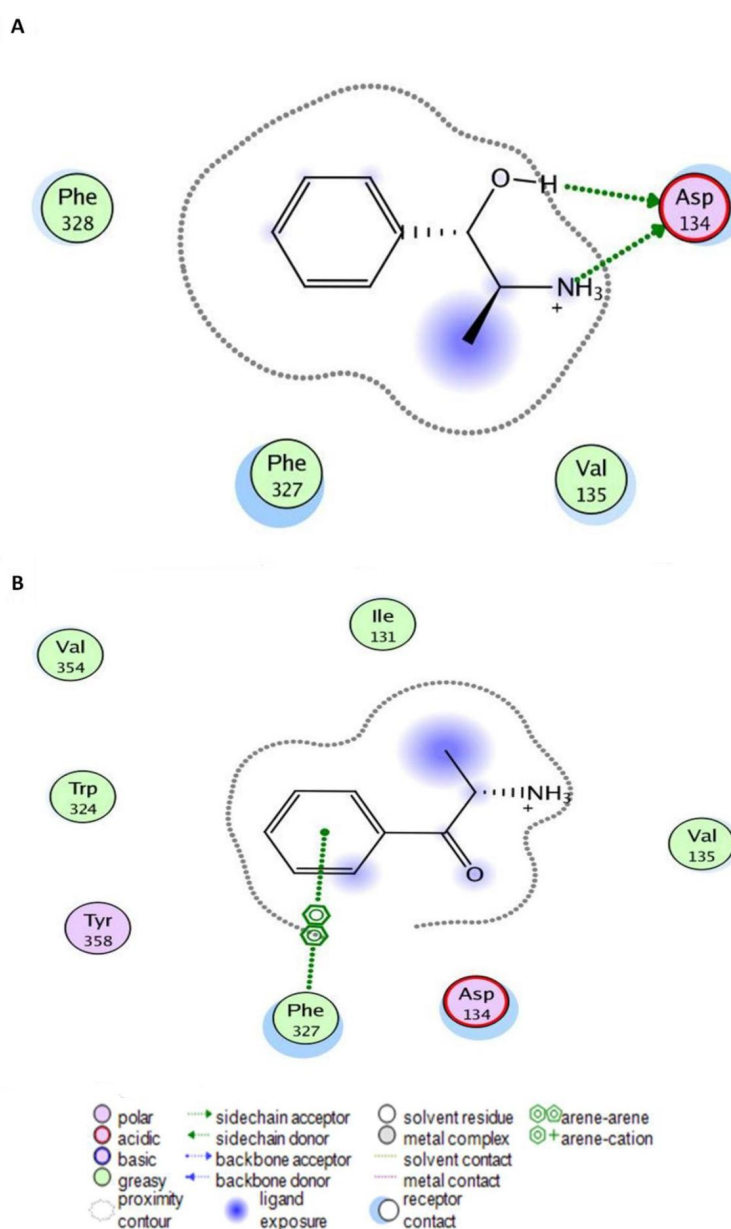

Figure 9 Cathine and cathinone interactions at the G-binding site. The 2D ligand-protein interactions of cathine and cathinone in the binding site of G (A and B) are indicated by green dashed arrows, indicating hydrogen bonds. The blue haloes show solvent-exposed atoms.
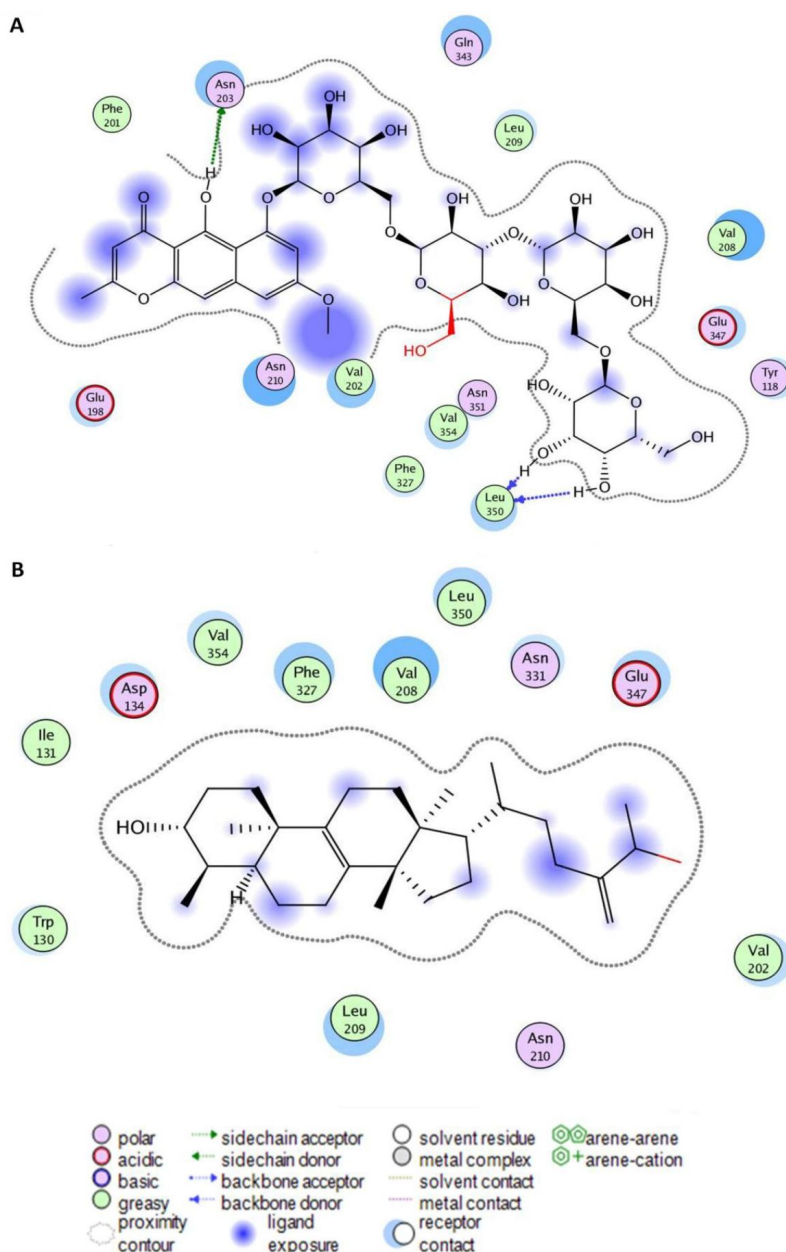
Figure 10 Cassiaside B2 and obtusifoliol interactions at the H-binding site. The 2D ligand-protein interactions of cassiaside B2 and obtusifoliol in the binding site of H (A and B) are indicated by green dashed arrows, which indicate hydrogen bonds. Blue haloes indicate solvent-exposed atoms.

in Figure 11A, indicates that the two residues interact via hydrogen bonding with a non-polar acidic side chain donor with Asp 134 and the polar side chain acceptor with Tyr 358. In addition, cathine interacted with the backbone donors Trp 130, Val 208, and Val 354, and a phenylalanine residue, Phe 327. Similarly, inspection of cathinone contacts with the H protein, as shown in Figure 11B, revealed four combinations of non-polar and polar residues and high ligand exposure to the aromatic and methyl groups. Interestingly, cathinone displays a hydrogen bond with the acidic side-chain donor Asp 117. In addition, there were contacts with the polar side-chain acceptors Asn 54, Gln 53, and Tyr 118.

Common residues interacting with Khat compounds. Figure 12, 13 show the residues that formed the most common contacts with the cathine and cathinone, respectively. As depicted in Table 3, a "1" marks the presence of a connection between the residue (rows) and the ligand (columns) if any atom of the residue or ligand lies within four angstroms of each other. The right-most column totals all these interactions for each residue and provides the total number of interacting molecules that come into contact with each residue.

Toxicity analysis and drug likeness. The toxicity of cathine and cathinone was assessed using the Protox-II webserver, which determined their carcinogenic, immunogenic, hepatotoxic, and cytotoxic properties. Table 4 presents the results of the LD_{50} toxicity class, indicating that cathine was found to be more toxic than cathinone. Cathine was classified as class 3, while cathinone was classified as class 4. Both compounds exhibited low gastrointestinal (GI) absorption. Interestingly, only cathinone showed a potential for carcinogenicity, whereas cathine did not exhibit this property. The results showed that the reference substances, cassiaside B2 and obtusifoliol, demonstrated carcinogenicity and immunotoxicity, respectively.

Discussion

This study aimed to investigate the role of Khat in appetite and body weight regulation using a comprehensive approach involving in vivo,

ex vivo, and in silico models. Notably, this study is the first to include both male and female rats and evaluate the effects of fresh Khat extract at different durations and doses. The study also focused on elucidating the impact of Khat on 5-HT receptors, which was explored using rodent fundus experiments and bioinformatics tools. To ensure the relevance of the research to practical applications, fresh Khat was used instead of dried Khat, as drying processes can reduce the concentration of active compounds [41]. Previous studies investigating the effects of Khat on appetite have been limited by the use of dried Khat, which may have compromised the accuracy of the results [5, 7]. To further validate the findings of this study, the plasma of rats treated with CEWE was analyzed using LC-MS/MS to quantify the levels of cathine and cathinone. LC-MS/MS is currently considered the most reliable method for detecting amphetamine-like compounds in research and forensic laboratories [42].

The objective of this study was to investigate the potential anti-obesity effects of CEWE in rats over a period of eight weeks. To ensure the reliability of the study, several measures were taken to maintain uniformity among the experimental conditions. Sprague-Dawley rats of the same age were selected, standardized cages were used, and consistent conditions of light, humidity, and temperature were maintained. These steps aimed to minimize variations in growth, development, physical activity, and environmental factors, thereby enhancing the internal validity of the findings and providing a more reliable experimental experience. The results showed that treatment with CEWE at doses of 100, 300, and 500 mg/kg led to significant reductions in body weight gain compared to the control group. These findings are consistent with previous research that explored the effects of Khat consumption on weight loss [4, 7, 41, 43]. Previous studies have consistently demonstrated that Khat administration in rat models results in decreased body weight. It has been observed that sustained weight loss occurs with higher doses or longer treatment durations, while lower doses may lead to

short-term weight loss followed by the development of tolerance to this effect [44]. Additionally, a cross-sectional study conducted among healthy individuals in Ethiopia found that Khat users had a lower mean body weight compared to non-users [45]. These collective findings support the potential of CEWE as a weight management agent and emphasize the importance of further investigation into its mechanisms and long-term effects.

Leptin and ghrelin are hormones known for their roles in regulating appetite. Activation of leptin or ghrelin receptors in the hypothalamus initiates signaling pathways that influence food intake [17, 21]. However, further studies in rodents and humans are necessary to determine how Khat specifically affects leptin levels. To date, only two studies have investigated this aspect in rats and humans, but they did not provide sufficient evidence to support the notion that Khat has a significant effect on lowering leptin levels. These studies may have varied in terms of extraction methods, treatment duration, or dosage. Both studies attempted to explain the influence of gut hormones and stomach emptying on changes in leptin secretion [20, 23]. In the present study, the administration of CEWE did not lead to significant changes in plasma leptin levels among the different experimental groups of rats. However, we did observe variations in hormone levels between male and female rats. It is important to note that leptin levels in the bloodstream follow a diurnal pattern and are regulated by factors such as sex, age, physical activity, and glucose intake [46]. Our study found no immediate changes in leptin levels following a two-hour Khat treatment, suggesting that Khat may not have short-term effects on appetite via these hormonal pathways. Similar findings have been reported in previous studies [23]. In contrast, our study also revealed that Khat did not have an impact on ghrelin levels, regardless of the dose or treatment duration. This finding aligns with a previous report by Murray et al., who administered fresh Khat to habitual users for three hours and observed no changes in blood ghrelin levels [23].

Table 3 Common ligand-binding residues of selected ligands

Residues	Cathine-G	Cathinone-G	Cassiaside B2-H	Obtusifoliol-H	Cathine-H	Cathinone-H	Sum
Asp 134*	1	1		1	1		4
Asp 117						1	1
Asn 54						1	1
Asn 203			1				1
Asn 210			1				1
Asn 331				1			1
Asn 351			1				1
Gln 53						1	1
Gln 343			1				1
Glu 198			1				1
Glu 347			1	1			2
Ile 131		1		1			2
Leu 209			1	1			2
Leu 250			1				1
Leu 350				1			1
Phe 201			1				1
Phe 327*		1	1	1			3
Phe 328	1						1
Trp 130				1	1		2
Trp 324		1					1
Tyr 358					1		1
Tyr 118			1			1	2
Val 135	1	1					2
Val 208*			1	1	1		3
Val 202			1	1			
Val 354*			1	1	1		3

1: the presence of a contract between the residues (rows) and the ligand (columns) if any atom of the residue or ligand lies within 4 angstrom. * residues of remarkable importance.

Table 4 Absorption, distribution, metabolism, excretion, toxicity prediction

Compound	Lipinski	GI absorption	BBB permanent	LD_{50} class	Predicted LD_{50} mg/kg	Carcinogenicity probability	Immunotoxicity	nrf2/ARE	HSE
Cassiaside B2	No; 3 violations: MW > 500, N or O > 10, NH or OH > 5	Low	No	5	5,000	Active	Active	Inactive	Inactive
Obtusifoliol	Yes; 1 violation MLOGP > 4.15	Low	No	4	2,000	Inactive	Active	Active	Active
Cathine	Yes; 0 violation	High	Yes	3	150	Inactive	Inactive	Inactive	Inactive
Cathinone	Yes; 0 violation	High	Yes	4	400	Active – 0.64	Inactive	Inactive	Inactive

LD_{50} classes: Class I: fatal if swallowed ($LD_{50} \leq 5$ mg/kg), Class II: fatal if swallowed ($5 < LD_{50} \leq 50$ mg/kg), Class III: toxic if swallowed ($50 < LD_{50} \leq 300$ mg/kg), Class IV: harmful if swallowed ($300 < LD_{50} \leq 2,000$ mg/kg), Class V: may be harmful if swallowed ($2,000 < LD_{50} \leq 5,000$ mg/kg), Class VI: nontoxic ($LD_{50} > 5,000$ mg/kg). nrf2/ARE, nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element; HSE, heat shock factor response element; GI, gastrointestinal; BBB, blood brain barrier.
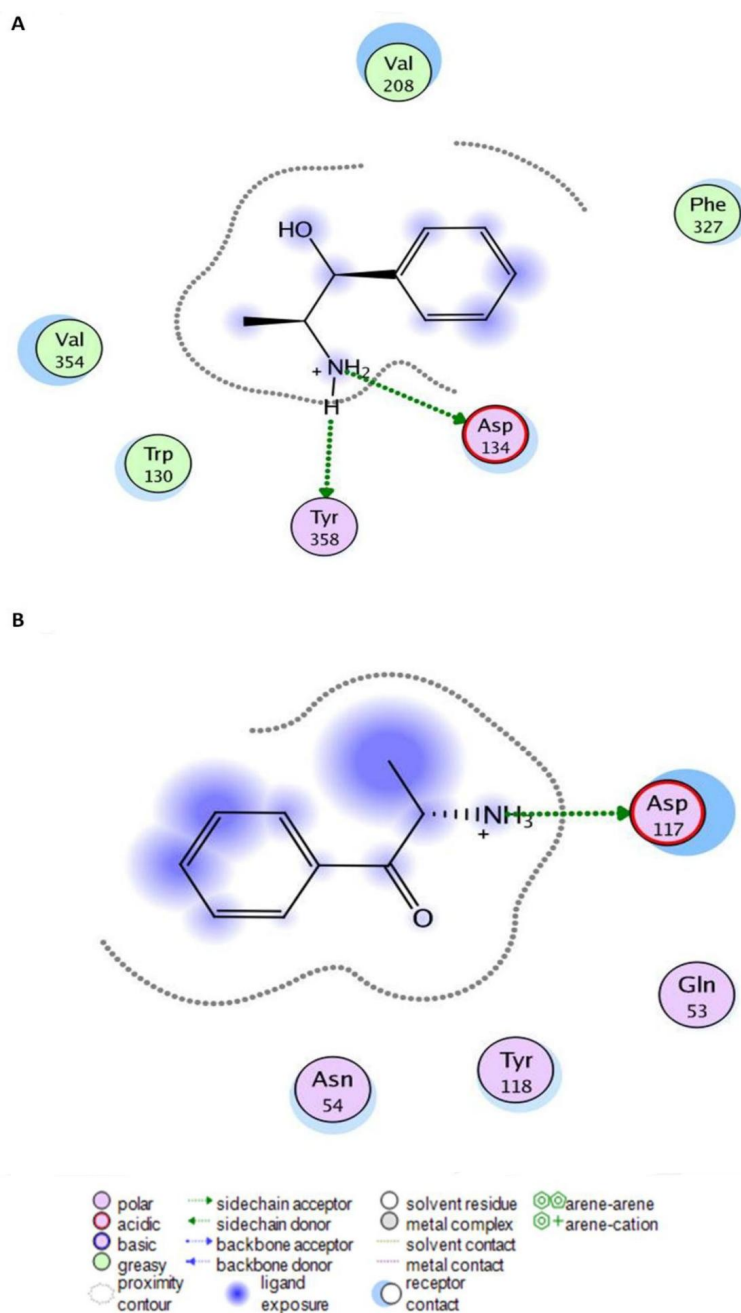

Figure 11 Cathine and cathinone interaction in the binding site of H. The 2D ligand-protein interactions of cathine and cathinone in the binding site of H (A and B) are indicated by green dashed arrows, which indicate hydrogen bonds. Blue haloes indicate solvent-exposed atoms.
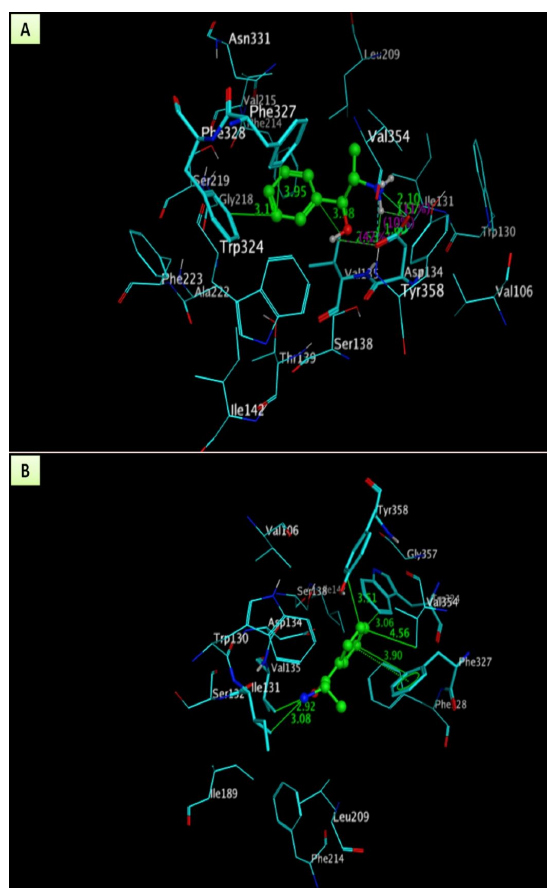
Figure 12 3D view of the interaction between cathine and cathinone in the binding site of G (A and B). The compounds are shown as green carbon balls, sticks, and residues as aqua sticks. The purple dashed lines indicate the hydrogen bonds labeled with their strengths and lengths.
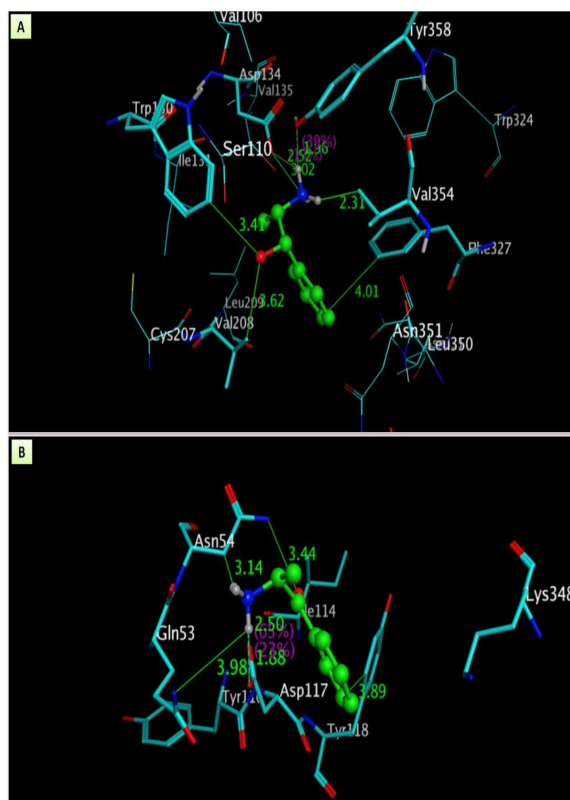

Figure 13 3D view of the interaction between cathine and cathinone in the binding site of H (A and B). The compounds are shown as green carbon balls and sticks, and the residues are shown as aqua sticks. The purple dashed lines indicate the hydrogen bonds, which are labeled with their strengths and lengths.

Serotonin (5-HT) is known to have a role in regulating appetite. It influences appetite reduction in the hypothalamus by increasing the levels of two anorexigenic peptides [47]. In rats, medications that affect 5-HT levels have been shown to decrease food intake, indicating increased feelings of fullness. These medications have also been proven to reduce calorie intake in humans, leading to decreased appetite and increased satiety. The 5-HT2C receptor subtype is particularly involved in these effects. Medications that act on 5-HT, such as fenfluramine, d-fenfluramine, and sibutramine, have demonstrated effectiveness as anti-obesity therapies [48, 49]. Khat, the plant under investigation in this study, also possesses properties related to serotonin. For instance, cathinone has been shown to increase serotonin release from the striatum in vitro, bind to serotonin receptors, and inhibit serotonin reuptake after a single or repeated dose [50]. In this study, Khat extract was found to antagonize 5-HT in rat fundus strips. The serotonin-antagonizing properties of Khat may explain its anorexic properties, as observed in this study. These findings support the proposed mechanism of Khat's appetite-suppressive action, which involves altering dopamine and serotonin physiology. Fenfluramine, a weight loss medication, suppresses hunger by releasing serotonin and dopamine into the striatum [51].

The simulation results indicated that cathine and cathinone bound to 5-HT2C receptors G and H. Their free binding energies were lower than obtusifoliol but higher than cassiaside B2. Molecular contact with the highly conserved residue Asp 134 was found to be important for agonist activity [52]. Cathine and cathinone interacted with the G protein through Asp 134 and established several polar and nonpolar hydrogen bonds with residues 3 and 6, respectively. However, these interactions did not match those of the reference compounds. Interestingly, these residues are known to be essential for the ligand-binding activity of small molecules, suggesting the potential agonist activities of cathine and cathinone [53].

Similarly, the ligands contacted the H protein through polar and nonpolar hydrogen bonds with six and four residues, respectively. Some of these residues, including Asp 134, Trp 130, Val 208, Val 354, Tyr 118, and Phe 327, are crucial for the antagonistic activity of standard ligands against the H protein. The predicted interactions typically involved a combination of ring-stacking with aromatic residues, supported by clusters of other direct hydrogen bonds and smaller non-polar, polar, and acidic residues. The study identified several key residues, including Asp 134, Phe 327, Val 202, and Val 354, that are involved in the appetite inhibition effect of cathine and cathinone. Taken together, the findings from ex vivo and in silico studies support the role of cathine and cathinone, components of Khat, as 5-HT antagonists, suggesting their potential involvement in appetite regulation.

In the current study, SwissADME, a free website, was utilized to investigate the pharmacokinetic properties of cathine and cathinone. This tool evaluates drug-like parameters based on Lipinski's rule of five, which is used to assess the absorption and permeability of a molecule across lipid bilayers in the human body [40]. Lipinski's rule states that a compound is more likely to be bioavailable if its molecular weight is below 500 Da, it has fewer than 10 hydrogen bond acceptors, fewer than five hydrogen bond donors, and a log P value (partition coefficient) lower than five [54]. Importantly, both cathine and cathinone adhere to Lipinski's rule. Interestingly, SwissADME also predicted that the GI absorption of cathine and cathinone is high, indicating that they have a greater propensity to effectively bind to the G and H receptors, potentially impacting human appetite. Compounds with higher absorption rates are generally more effective [55]. Additionally, the ligands were assessed for their ability to penetrate the blood brain barrier (BBB), which is crucial as 5-HT2C receptors are widely distributed in human brain tissue. They are expressed in regions such as the substantia nigra, ventral tegmental area, nucleus accumbens, striatum, and prefrontal cortex, which are associated with dopaminergic cell bodies and terminal projection areas [56]. According to SwissADME, cathine and cathinone exhibit high BBB penetration, suggesting that they can

readily and effectively interact with their targets, G and H receptors. Furthermore, this in silico study predicted the toxicity levels of cathine and cathinone using the Protox website, which revealed low LD_{50} values. This finding aligns with the classification of Khat as a class C drug by the World Health Organization [57]. Prolonged consumption of Khat has been associated with increased cancer risk in the human body due to cellular oxidative stress, attributed to the ability of Khat leaves to undergo naturalization in the gut and produce nitrosamines [58].

Conclusions

In conclusion, this study successfully measured the active components of Khat in rat plasma using liquid chromatography-mass spectrometry. The water extract of Khat was found to decrease rat weight without affecting the levels of appetite-related hormones leptin and ghrelin and their associated mRNA. Ex vivo, RT-qPCR, and in silico experiments demonstrated the antagonistic effects of Khat on 5-HT receptors, suggesting its potential as an appetite suppressant through central serotonergic activity. This study also provided initial insights into the pharmacokinetics and toxicity of cathine and cathinone. However, further research is needed to validate the appetite-suppressing effects of Khat, explore its therapeutic applications, evaluate its toxicity comprehensively, investigate underlying mechanisms, and raise public health awareness. These recommendations include additional empirical studies, clinical trials, computational assessments, mechanistic investigations, comprehensive toxicity evaluations, collaborative research efforts, and public education initiatives. Future studies could consider incorporating techniques such as western blotting and immunofluorescence to strengthen and validate the findings. Overall, further investigation is required to unlock the full potential of Khat and its components for appetite regulation and other therapeutic purposes.

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