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Full Length Research Paper

Interaction between pro-inflammatory cytokines and brain oxidative stress biomarkers of khat, cathinone and pseudoephedrine hydrochloride intoxication in male mice

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Cathinone is a potent central nervous system (CNS) stimulant found in khat leaves. Cathinone and synthetic cathinone have become popular recreational drugs in recent years, commonly marked as "legal highs". Pseudoephedrine more commonly known as cathine closely related to phenyl alkylamines that have been isolated from the *Catha edulis* "khat". The present study aimed to examine the potency differences between cathinone, khat extract and pseudoephedrine through evaluating the main brain oxidative stress biomarkers and pro-inflammatory cytokines. The mice were divided into four groups according to oral treatments for two weeks as: 1- control, 2- khat methanol extract (500 mg/kg bwt) -3- cathinone (1.6 mg/kg bwt) and 4- pseudoephedrine (10 mg/kg bwt) animals decapitated after two intervals, one and two weeks of treatments and whole brain tissues used for determination of total glutathione (GSH), oxidized glutathione (GSSG), GSH/GSSG ratio, nitric oxide (NO), calcium ions, uric acid, malondialdhyde (MDA), cytochrome P450 (CYP 450), interleukin 1 β (IL1β), interleukin 6 (IL6), tumor necrosis factor α (TNFα), and cyclooxygenase 2 (COX2). The results revealed an increase in oxidative stress and brain cytokines through increasing the investigated parameters and decreasing of GSH, GSH/GSSG ratio and Ca in treated groups in comparison with the control. The results suggested the responsibility of treatments on the pathophysiological changes in brain tissues that related to the existed oxidative stress and cytokines.

Key words: Khat, cathinone, pseudoephdrine, cytokines, oxidative stress.

INTRODUCTION

Khat "*Catha edulis* Forsk" is an evergreen shrub of the "Celastraceae" family. It is widely cultivated in Yemen and East Africa, where its fresh leaves is habitually chewed for their momentary pleasures and stimulation as amphetamine-like effects (Atlabachew et al., 2013). Moreover, it is estimated that there are at least 10 million

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people globally who chew khat leaves on a daily basis (Odenwald et al., 2010) with evidence of the spread of cultivation and use khat in regions outside of the traditional area (Klein and Metaal, 2010). Khat chewing is linked with increased oxidative stress (Aleryani et al., 2011). The oxidative stress in restrained rats was noticed due to the decreased activity of antioxidant enzymes, superoxide dismutase, catalase and glutathione-S-transferase after oral administration of total aqueous Khat extract or of its alkaloid fraction (Kalix, 1990). Similarly, an increase in reactive oxygen species (ROS) and a depletion of intracellular glutathione in the cell cultures of human keratinocytes and fibroblasts after Khat treatment was observed. However, the addition of exogenous antioxidants opposed the reactions (Lukandu et al., 2008).

Cathinone is the principal active psychostimulant constituent of the leaves of the khat shrub responsible for the stimulant effects that led khat being known as "natural amphetamine". Derivatives of cathinone have recently come to prominence as "legal highs" and have been the subject of intense interest resulting in restrictive legislation introduced in a number of countries (Kelly, 2011). The alkaloid cathinone represents its main psychoactive compound, with a structure and pharmacological profile similar to synthetic drug amphetamine (Kalix, 1990). In addition to cathinone and its derivatives, khat contains other bioactive constituents, including tannis, ascorbic acid, the phenylalkylamine (Brenneisen, 1984), flavenoids and triterpenoids (Kalix, 1990).

Pseudoephedrine (PS) salts, hydrochloride and sulphate are steroisomer of ephedrine that produces mixed adrenergic agonist effects and commonly used as nasal and para nasal decongestants by scuba divers (Pilla et al., 2013) and as the treatment of common cold symptoms. Pseudoephedrine is a sympathomimetic drug and psychostimulant (Hoffman and Lefkowitz, 1996) and belongs to the family of phenylethylamines and amphetamines (Hoffman and Lefkowitz, 1996; Kobayashi et al., 2003). Its primary action, vasoconstriction results from direct and indirect activation of α-β- adrenergic receptors, causing reduction of nasal membrane inflammation and mucus production.

The aim of this study was to compare between the khat methanol extract or cathinone and the pseudoephedrine hydrochloride administration through studying the brain oxidative stress biomarkers and pro-inflammatory cytokines. Previous studies discussed the massive force of the aforementioned administered materials on the monoamine systems (Patel, 2000; Simmler et al., 2013) and liver (Chapman et al., 2010). Nevertheless, the brain cytokines was not discussed in the literature work. Hence, we aimed to compare a small dose of the methanol khat extract or its main active substance cathinone as a treatment for a short time "one or two weeks" to reveal the neuroinflammation toxicity in comparison with the synthetic drug pseudoephedrine hydrochloride.

MATERIALS AND METHODS

Experimental animals

The study used forty-eight adult male Swiss albino mice weighing 20 to 25 g from the Egyptian Organization for Vaccine and Biology preparations at Helwan, Cairo. The animals were housed in groups of twelve in plastic cages and bedded with wood shaving. They were kept under hygienic, aseptic condition and fed on standard rodent chow and fresh tap water *ad libitum*. Mice habituated to the testing ground conditions for one week prior to the experiment. The experimental protocol and procedures was approved by Ain Shams University authorities and followed Egyptian rules for animal protection that is in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November, 1986 (86/609/EEC). Efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

Preparation of Khat (*Catha edulis*) extract (methanol extract)

Khat extract was prepared by first washing fresh khat leaves with distilled water, and then 90.74 g of fresh leaves crushed with pestle and mortar. After crushing, 800 ml of methanol was added, stirred gently and then allowed to stand overnight. Filtration was done and then the methanol was evaporated using a Rotavac control evaporator. The remaining extract was weighed as 32.5g and used to prepare a working concentration (Kimani and Nyongesa, 2008).

Extraction of cathinone

The method of extract by Mohammed and Engidawork (2011) was used. Cathinone was isolated from fresh *Catha edulis* leaves, soaked in 0.1 N HCl, sonicated for 30 min, then filtrated, and then extracted using diethyl ether. The aqueous acid layer was basified with 10% NaOH, and final extraction with diethyl ether. To the diethyl ether extract saturated oxalic acid solution was added drop wise, and left to stand for 20 h in the refrigerator to yield cathinone oxalate as white precipitate.

Experimental design

Male albino mice was divided into four groups, each of the twelve was treated with oral tube daily as follows: the first was the control (Con) which received distilled water. The second group (Kt), treated with Khat extract and received 500 mg/kg b.wt dissolved in distilled water (Al-Hashem et al., 2012). The third group (Ca) was administrated cathinone extract at a dose of 1.6 mg/kg body weight dissolved in distilled water (Brenneisen et al., 1990). The fourth group (Ps), treated with 10 mg/kg b.wt pseudoephedrine hydrochloride dissolved in distilled water (Reagan-Shaw et al., 2008). All groups received treatments daily for either one or two weeks.

Biochemical assay

After one and two weeks of treatment, mice was sacrificed 12 h following the last dose by rapid decapitation. Brains were excised on ice and then homogenized in icy cold buffer saline. Brain supernatant was used for the determination of the reduced and oxidized glutathione using ELISA Kit (Oxford Biomedical Research, Germany). Total nitric oxide (NO) was determined using ELISA Kit specific for mice assays (Assay Designs, Inc-Germany). Brain free

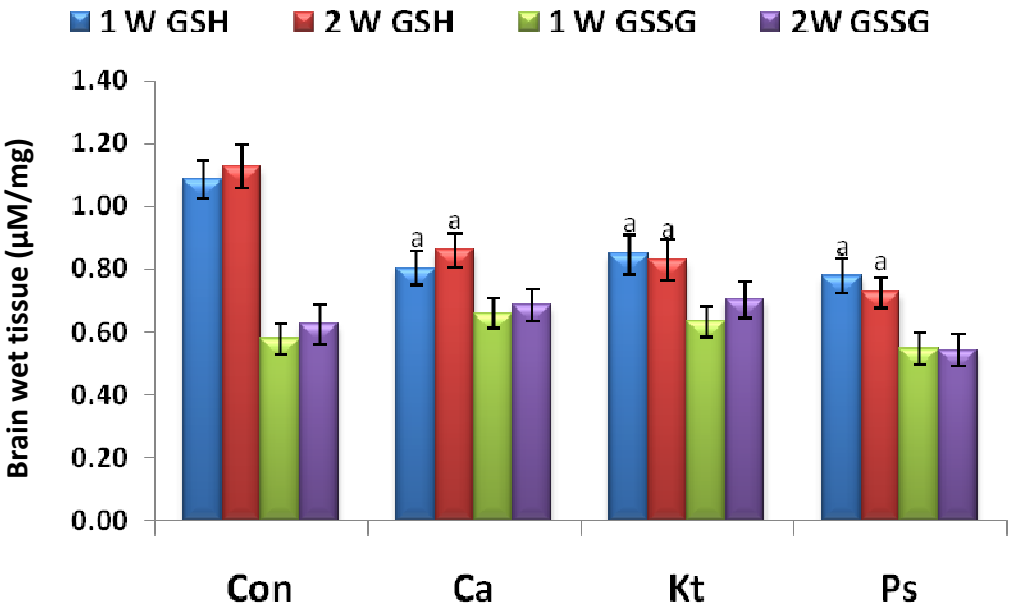

Figure 1. The GSH and GSSG content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

ionic calcium and uric acid contents was determined using calorimetric kits (Diachem Ltd, Hungary). Malondialdehyde content was determined using a calorimetric kit (Biovision - USA). The activity of brain total cytochrome P450 (CYP 450) was determined by using ELISA Kit (Life Science, Inc-Germany. The contents of brain interleukin -1β (IL-1 β) (cod no. 27193), interleukin-6 (IL-6) (cod no. 27768), tumor necrosis factor alpha (TNF-α) (cod no. 27194) and brain cyclooxyenase (COX-2) (cod no. 27187) was determined using enzyme immunoassay (EIA) techniques (IBL, Japan). All the used kits are specific for mice.

Statistical analysis

Reported values represent means of six mice ± standard error (SE). Statistical analysis was evaluated by one-way analysis of variance (ANOVA). Once a significant F-test was obtained, least significant difference (LSD) comparisons was performed to assess the significance of differences among various treatment groups. Statistical package for social science "SPSS" for Windows software, Release 20.0 (SPSS, Chicago, IL) was used.

RESULTS

The study showed significant decrease in glutathione (GSH) and GSH/GSSG ratio in all treated groups throughout the two experimental periods in comparison with the control (Figures 1 and 2), while the total cytochrome P450 (CYP450) significantly increased in the cathinone and pseudoephedrine groups after one week of treatment as compared with the control group (Figure 3). Moreover, after two weeks of treatment only pseudoephedrine treated mice exhibited a significant increase in CYP450 as compared to the control ($p \geq 0.05$).

Nitric oxide, MDA and uric acid contents increased significantly in all treated groups as compared to the control throughout the two experimental periods, except khat group after two weeks of treatment. However, all the treated mice exhibited a significant decrease in brain free Calcium content decreased significantly in all groups except the cathinone treated group for one week as compared to the control (Figures 4 to 7).

Figures 8 to 11 showed that there were significant increases in IL-6 and TNF-α in all treated groups after one and two weeks of treatments as compared with the control group. IL-1β content significantly increased in cathinone treated group after one week and khat treated group throughout the experimental intervals in comparison with the control values. All the treated groups displayed a substantial increase in COX-2 after one-week of treatment as compared to the control value, while cathinone and pseudoephedrine treated groups showed significant increase in COX-2 after two weeks compared with the control values.

DISCUSSION

The results of the present study demonstrated that treatment with cathinone, khat extract or pseudoephedrine significantly increased brain oxidative

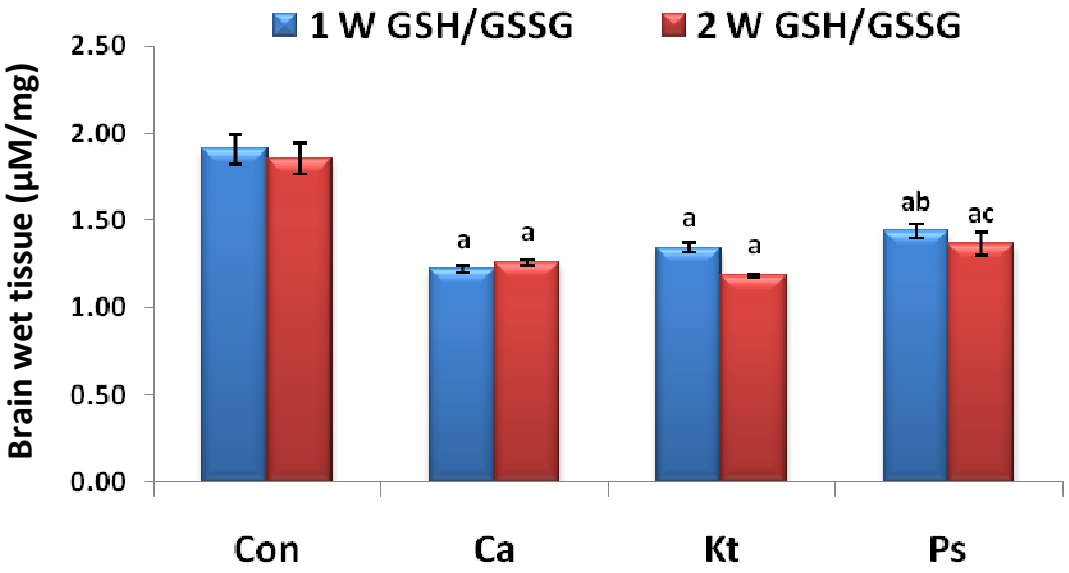

Figure 2. the GSH/GSSG ratio content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

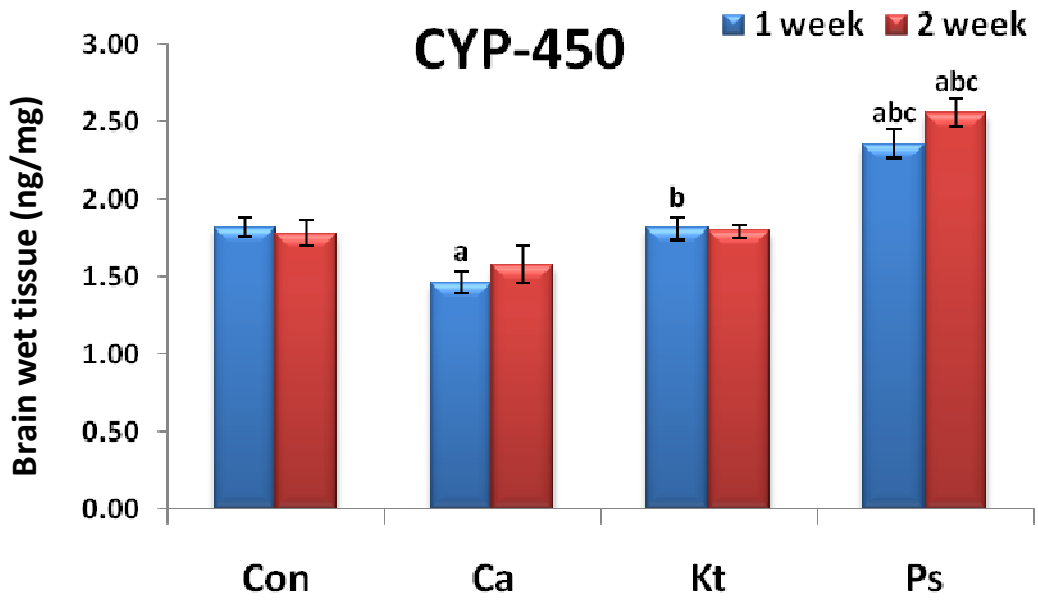

Figure 3. the total CYP-450 activity in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

stress markers. Previous studies revealed oxidative stress of khat or its components through significant increases in lipid peroxidation biomarkers thiobarbituric acid reactive substances (TBARS) and significant decreases in levels of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) (Al-Akwa et al., 2009; Al-Hashem et al., 2011; Masoud et al., 2012). Al-Hashem et al. (2011) indicated that orally administered *Catha edulis* extract exerts severe hepato-nephro toxicity and the mechanism of this damage may be related to

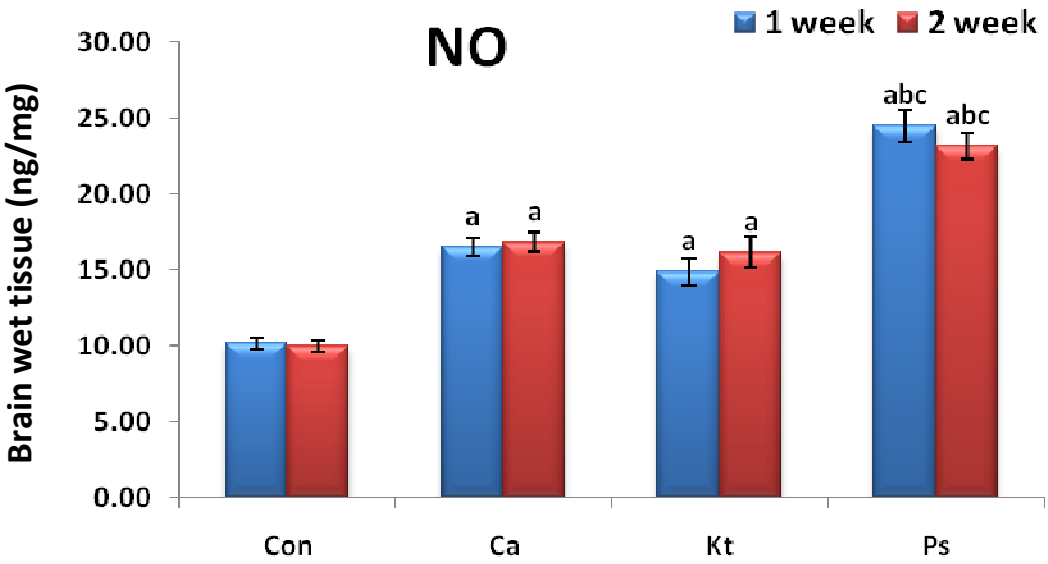

Figure 4. The total nitric oxide NO in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

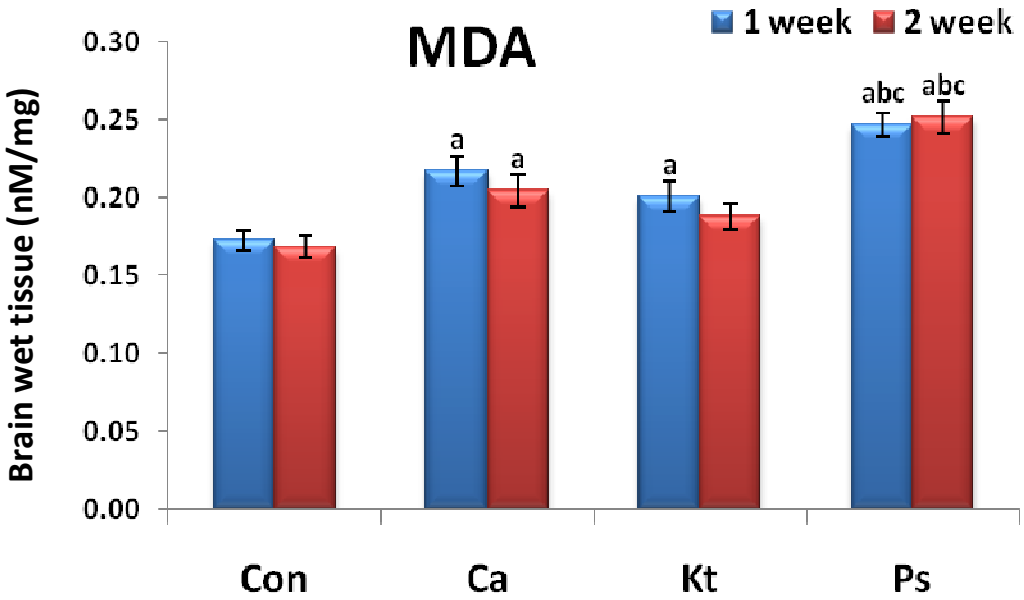

Figure 5. the MDA content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

oxidation, increased lipid peroxidation, and generation of free radicals inside these tissues.

In the present study, low levels of GSH could be due to the enhanced generation of ROS revealed by increasing MDA which are scavenged by GSH or decreased activity of enzymes in all treated groups, which converts oxidized glutathione (GSSG) to its reduced form. The GSH/GSSG ratio or glutathione redox status considered index of the

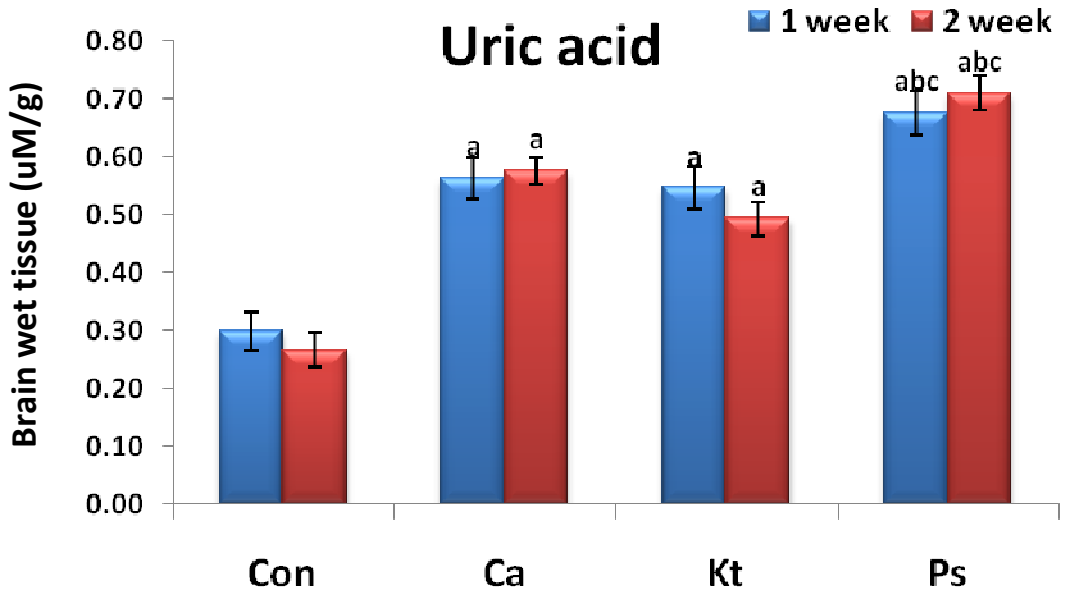

Figure 6. the uric acid content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c = significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

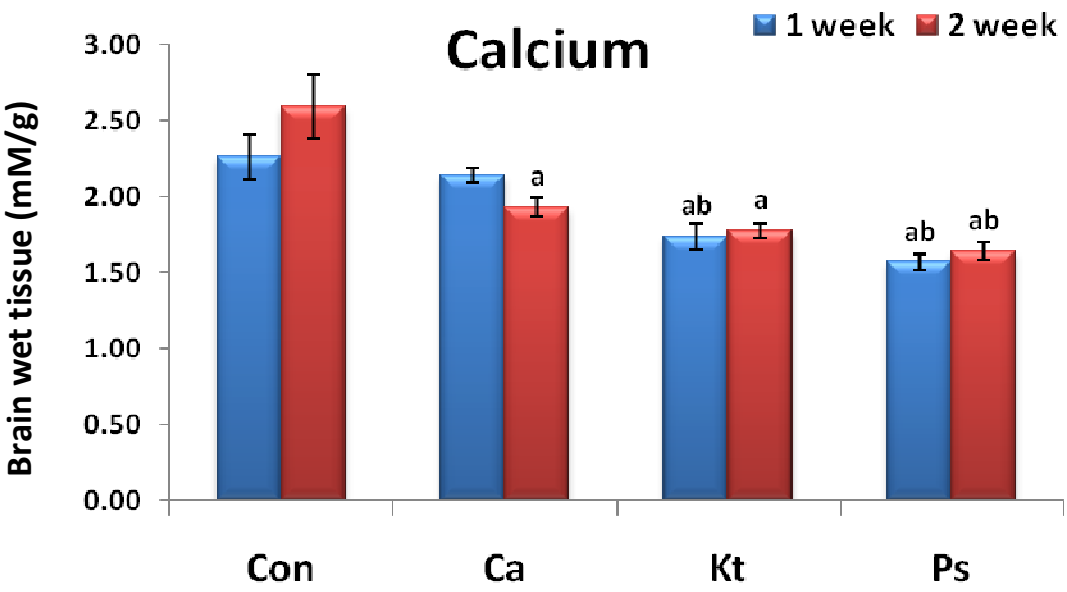

Figure 7. the free calcium ion in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

cellular redox status and a biomarker of oxidative damage because glutathione maintains the thiol-disulphide status of proteins, acting as a redox buffer. Also, glutathione depletion induced apoptotic cell death which occurs through the upregulating novel protein kinase C and activator protein-I (Domenicotti et al., 2000). The decrease in glutathione antioxidant system might relate to what was previously reported as cathinone

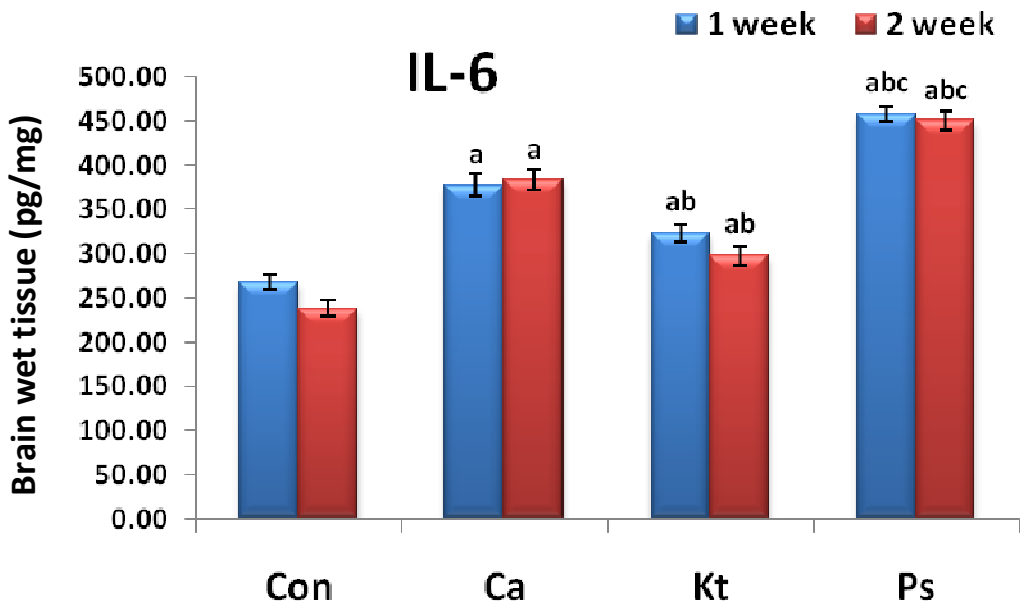

Figure 8. the IL-6 content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca = cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

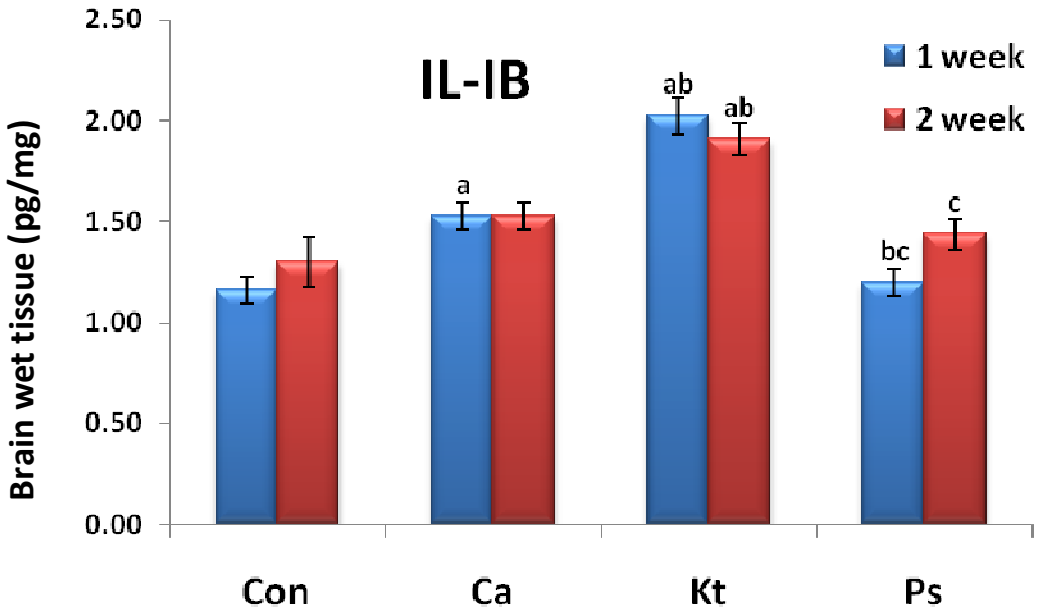

Figure 9. The IL-1β content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

and khat like methamphetamine increases the auto-oxidation of dopamine, leading to increase singling of oxidative stress (Feyissa and Kelly, 2008; Zhu et al., 2006). Moreover, Yao et al. (2013) suggested that alternation in the key serotonin pathways might contribute to oxidative stress via purine catabolism (uric acid,

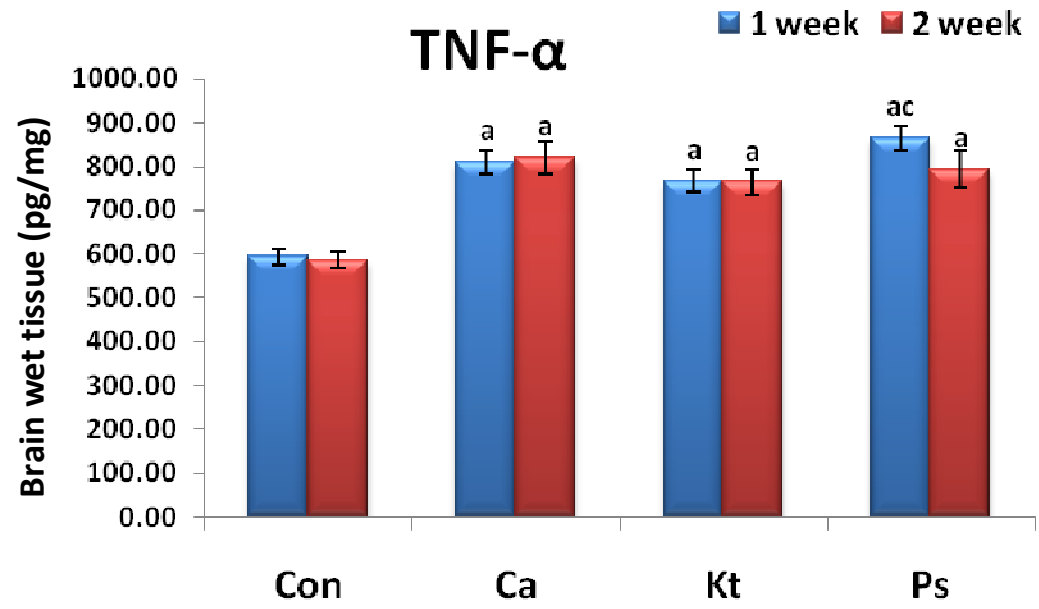

Figure 10. The TNF-α content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

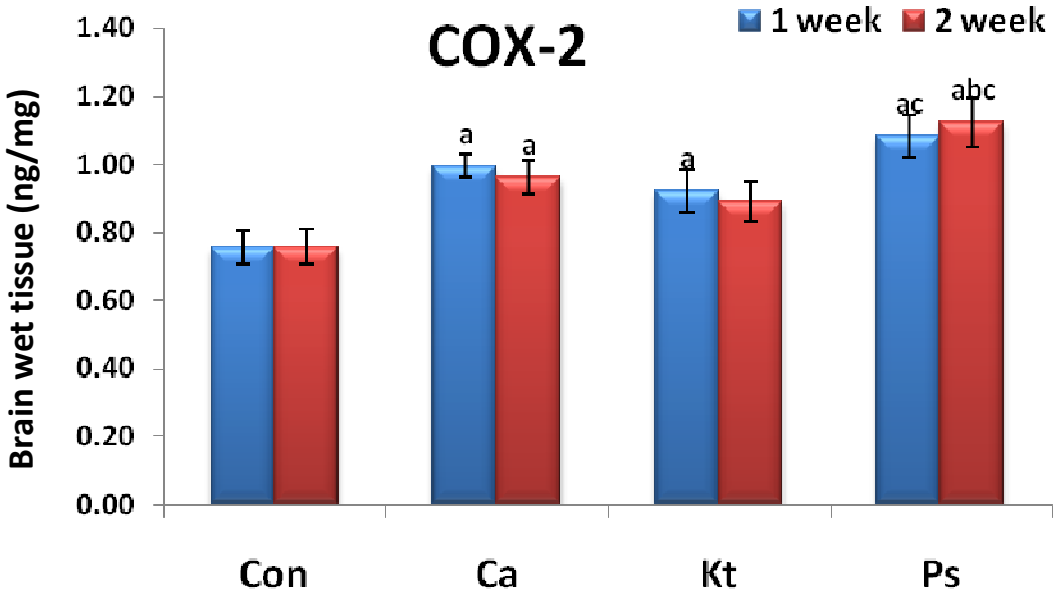

Figure 11. The COX-2 content in the total brain tissue of different treated groups. Values are means± SE of 6 mice at p≤0. 05, a=significant change from corresponding control, b=significant change from cathinone, c= significant change from khat, Con= control, Ca= cathinone, Kt= khat and Ps= pseudoephedrine hydrochloride.

guanosine or xanthine). The decline in GSH paralleled with elevation in brain lipid peroxidation and NO contents. These results could be explained as reported that in the human brain, NO is metabolized primarily in the form of nitrate. Because the reaction of NO with free thiols competes with some substrate (GSH), the excessive NO formation may further lead to a significant depletion of GSH in schizophrenia (SZ) (Yao et al., 2013). Treatment

with cathinone, khat extract or Ps causes increase in oxidative stress status, which may resemble the effect of psychiatric disorders such as SZ.

Uric acid at high levels is considered as a marker of oxidative stress in the brain (Becker, 1993; Strazzullo and Puig, 2007) due to the accumulation of reactive oxygen species (Hayden and Tyagi, 2004). Our study recorded highly significant increases of uric acid contents in all treated groups. Abnormally elevated levels of uric acid is tied to cardiovascular disease, gout, high blood pressure, and renal disease (Kang et al., 2002; Choi et al., 2005; Bos et al., 2006). Moreover, cathinone, khat and Ps could diffuse into the mitochondria like methamphetamine and build up positively charged particles in the mitochondria interferes with the chemical gradient needed by the electron transport chain displacement of Ca^{+} ions stored in the endoplasmic reticulum leading to influx into mitochondria. The release of those ions results in the activation of pro-apoptotic factors and disrupts cellular metabolism (Davidson et al., 2001; Feyissa and Kelly, 2008). There is a link between uric acid content in the brain and calcium ion level. Uric acid may work as a protective factor mediated through astroglia for dopaminergic neurons from glutamate toxicity (de Lau et al., 2005; Du et al., 2007). Moreover, uric acid prevents the propagation of oxidative stress from the extra- to intracellular location by protecting the integrity of the plasma membrane at the lipid-aqueous interface border (Guerreiro et al., 2009). Uric acid provides a neuro-protection by increasing Ca^{2} and extracellular signal-regulated kinases ½ (ERK1/2) through amplifies K^{+}-induced free calcium ion level reflects the oxidative stress status represented in all treated groups. A correlation between elevated lipid peroxidation, uric acid levels, and total NO content in total brain tissues and the reduction of GSH and GSH/GSSG ratio is observed in this survey.

The elevated brain cytokines because of activation of the brain microgalial cells can be beneficial in the protection of cells, but it can also release neurotoxic cytokines, which could have a deleterious effect and produce neurodegeneration (Liu et al., 2003). As shown by the results, our data indicated that cathinone, khat extract and PS groups after one and two weeks was found to cause a significant increase in IL-1β, IL-6, TNFα and COX-2 along with a significant increase in NO level in the whole brain tissues compared to control. Moreover, IL-1 induces release of the inflammatory mediator IL-6 from galia and neurons through type one-receptor IL-IRI singling (Lee et al., 1993; Ringheim et al., 1995). Interleukin 6 concentrations tend to be more readily detectable and more consistent over time. The present study revealed that the induction of cyclooxygenase (COX2) in all treated groups suggested causing neuroinflammatory process resulting in deleterious events in the cell (Zhang et al., 2007).

In the present study, the increase in IL-1β and IL-6 have modulator effects on electrophysiological properties of thalamic neurons, implying that the thalamic functions may be affected by systemic disorders that present with high levels of those cytokines (Samios and Inoue, 2014). Previous studies showed that increase in the IL-1 release contribute to neurodegeneration in both acute and chronic brain conditions, findings from acute ischemia, stroke and brain injuries, while, the release of IL-1 and other proinflammatory cytokines, such as TNF-α, may protect neurons (Tuttolomondo et al., 2008; Shin et al., 2009). Song et al. (2006) suggested that IL-1 enhances the responsiveness of rats to stressful environmental stimuli. In addition, the sub-chronic administration of IL-1 induces behavioral, neurotransmitter; hormonal and immune changes may be implicated in the mechanism of some of psychiatric disorders such as depression. Therefore, administration of IL-1 to rodents is used as a model for studying the interaction between inflammation, brain functions, and memory deficits in neurodegenerative and psychiatric diseases (Patel et al., 2006).

Conclusion

Oxidative stress, inhibition of electron transport chain and inflammation all play a significant role in khat extract, cathinone and pseudoephedrine toxicity. No previous studies discussed the brain cytokines and oxidative stress effect on khat, cathinone and pseudoephedrine users. The synthetic cathine "Pseudoephedrine" could be a better choice for treatment than khat extract or cathinone because it did not have an addiction effect on monoamine system and as shown in the present study it has the least effect on IL-1β content "the powerfully concerned in sterile inflammatory response".

Conflict of Interest

The authors have not declared any conflict of interest.

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