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METHANOL FRACTIONATIONS OF *CATHA EDULIS* FROSK (CELASTRACEAE) CONTRACTED LEWIS RAT AORTA IN VITRO: A COMPARISON BETWEEN CRIMSON AND GREEN LEAVES

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Abstract

The study investigated the effect of methanol extract and its fractionations obtained from Yemeni khat on the smooth muscle isometric tension in Lewis rat aortal ring preparations and compared the effects of the crimson and green leaves. Khat leaves were sorted into green (khat Light; KL) and crimson (khat Dark; KD) leaves, extracted with methanol, followed with solvent-solvent extraction (benzene, chloroform and ethylacetate). The contractile activity of the fractions was tested using aortal ring preparations. The control (phenylepherine contraction) methanol extracts contracted aortas at concentrations 250, 125 and 67.5 µg /1 ml buffer by 80.2% , 57.3%, 26.4% and 81.5%, 65.6% , 24.6% for KL and KD, respectively. Fractions of benzene (BF) and ethylacetate (EaF) contracted the aorta with 2µgm, whereas, chloroform (ChF) with 1 µgm / 1 ml buffer was less potent. The shape of contraction curve produced by EaF differed from that of ChF and BF of both (KL and KD). The EaF induced-contraction peaked after 3.3 ± 0.94 mins, whereas those of BF and CHF peaked after 18.0 ± 2.2, 19.7 ± 0.94 mins, respectively. Pre-incubation with nifedipine (10^{-6} M) insignificantly reduced the contraction induced by all fractionations, but prazosin (10^{-6} M) reduced the contraction by 81.9%, 63.1%, 71.8% with p= 0.23, 0.09, 0.15 for BF, ChF and EaF of KL, respectively. It significantly reduced contraction of ChF, 64.1%; p= 0.02, and of EaF, 73.5%; p= 0.04 of KD, while the reduction in contraction of BF was 63.1%; p= 0.06. In conclusion, fractions of green and crimson Yemeni khat leaves contracted aortas of Lewis rats. Both leaves behave almost similarly. Contraction induced by chloroform fraction produced alpha-sympathetic activity.

Key words: *Catha edulis*, aorta contraction, rat, cathinone.

Introduction

Catha edulis Frossk, simply called khat, is an evergreen shrub whose fresh leaves are habitually chewed for their central stimulating effect (Kalix, 1990; Fathala, 1991; Nencini, et al., 1989). Varieties of khat can be found in East Africa and Yemen, where its use is deeply anchored on the Yemeni tradition**.** Studies demonstrated that the composition of the khat is very complex. Although the phenylalkylamine group, cathinone (Anonymous, 1975; Kalix, 1983 and 1992) and cathine (Alles et al, 1961; WHO Advisory Group, 1980;) were well characterized, prenylalkylamines and cathedulines consisting of a mixture of 62 alkaloids (Kite, et al, 2003) need to be further studied. The effects of khat on the cardiovascular system became a source of growing concern

because some sensitive chewers may experience episodes of increased blood pressure during khat chewing. A study done on Yemeni healthy adult volunteers provided evidence that khat chewing produced a significant rise in arterial systolic and diastolic blood pressure and pulse rate. Most of the studies attributed the pharmacological actions of khat to cathinone (Kalix, 1984 and 1988; Brenneisen, et al., 1990; Calcagnetti and Schechter, 1992), but its precise mechanism of action needs to be elucidated. Other components of the leaves were overlooked. The present study was carried out to investigate the effect of methanol extract obtained from Yemeni *Catha edulis* and its fractionations on the smooth muscle preparations of the aortal ring preparations in Lewis rats. A study done by Al-Motarreb and Broadley (2004) showed that cathinone was not as potent as epinephrine on guinea pig aortas.

Several studies utilized undifferentiated chewable leaves (smooth crimson and green). Data of such works were incoherent. Therefore, we separated the chewable leaves of the same shrub. After drying, the green leaves became light green (khat light; KL) and the crimson leaves dark green (khat dark; KD). We attempt through this designed study to compare these leaves and highlight the possible mechanisms underling the action of the fractions.

Materials and methods

Plant materials

Fresh khat leaves (voucher number 06/2006) harvested in July 2006 from mountain Gehaf (Aldhalea province) were sorted according to colors within the young shrub into small, smooth, crimson (red) and small, smooth, green leaves. The leaves were air died. After drying, the crimson leaves became dark green and the other leaves light green. Then they were pulverized, transported in plastic bags to laboratories of the Institute of Pharmacy, University of Greifswald, Germany, and used within 2 weeks from harvesting. For the purpose of this study, the light green is given the name "khat light" (KL) and the dark green the name "khat dark" (KD).

Drugs

The following substances were used: Prazosin, phenylepherine and nifedipine were from Sigma Chemical, Saint Louis, Missouri, USA. Ninhydrin (for detection of free amino groups) and DPPH (1,1-Diphenyl-2-picrylhydrazyl-radical, for antioxidant assay) were also from Sigma-Aldrich. All solutions were prepared using distilled water except nifedipine that was first dissolved in ethanol and then in distilled water.

Extraction

20 g of the dried, pulverized leaves of both types were extracted with methanol for 15 hours using Soxhlet apparatus. The methanol extract was evaporated in a rotavapor and lyophilized. A definite amount (2 g) was dissolved in water and mixed with benzene. The extraction with benzene was done for six hours and repeated three times (3 x 6 hrs) on a shaker at room temperature. The benzene layer was evaporated to dryness and weighed. The remaining water layer was extracted with chloroform 3 x 6 hrs on a shaker at room temperature. After this the chloroform layer was evaporated to dryness and weighed. The residual water layer was mixed with ethyl acetate for 3 x 6 hrs on a shaker and the ethyl acetate layer was dried in a rotavapor and weighed. The water layer was removed.

In vitro experiments

Animals

15 male Lewis rats (LEW.1A, 220-280g) were obtained from the Department of Laboratory Animal Science (Karlsburg), Greifswald University. All animal treatment and experimental procedures were in accordance with the local Instructions for Animal Care of Greifswald University and the state animal care authority approved the project. Rats were housed in individual cages at a 12 hr light/dark cycle and received food and water *ad libitum*.

Aortal Contractility

Rats were anaesthetized with 60 mg thiopental-sodium per kg body weight and then the aorta was rapidly excised and immersed in Rat Modified Krebs-Henseleit buffer (K-H (in m M): NaCl 113, KCl 4.8, MgSO4 1.3, KH_2PO_4 1.2, NaH2CO3 25, $CaCl_2$ 2.5, glucose 7). Loose fat and connective tissue were carefully stripped. In some preparation endothelium was removed mechanically by gently rubbing the interior of the aortal preparation with a moistened cotton-wrapped metal stick. The aorta rings (2-3 mm long) were suspended

vertically between two stainless steel stirrups in the organ chambers filled with 20 ml of the control solution (K-H, 37ºC and pH 7.4) and bubbled with 95% O_2 and 5% CO_2. One of the stirrups was anchored to the rigid holder and the other upper one was connected to the isometric force transducer (ITI/25, EMKA Technologies, Paris, France), which was attached to micromanipulator, permitting precisely controlled displacement of the upper stirrup along strict vertical axis. The signal was amplified (STA 2808, EMKA Technology) and displayed on paper record (Rekadenki multipen record, R-50 series, Hugo Sachs Elektronik, Germany). After a period of stabilization (60 mins), the vascular muscle was stretched to its optimal length, which was established, in the preliminary experiments, to correspond to a counter - weight of 2g. The K-H solution (at 37°C, pH 7.4, gassed with 95% O_2/5% CO_2) was exchanged every 20 min, three times after each experiment. A test contraction with phenylepherine (10^{-7} M) was first produced. Once the plateau of the contraction elicited by phenylepherine was obtained, the aortic rings were rinsed with Krebs three times. After that, the khat extracts were added to detect the effect on the vascular tissue. The observation time was 30 mins (started by injection of the sample and ended with the steady state contraction). Effect of the khat extracts following pre-treatment of the preparations (20 mins pre-incubation) with nifedipine or prazocine were also investigated. The results are expressed as mean ± SEM. Student's-test was used for comparisons of the obtained mean values and significance set to $P<0.05$.

Results

The khat extracts

The yields of methanol extracts were 4.7 and 4.1 g for KL and KD, respectively. The final yields of the fractions are shown in Table 1. Thin layer chromatograms (TLC) using chloroform and methanol as a mobile phase (in ratio 9:1) qualitatively revealed the presence of alkaloids, cathinone and cathine as well as norephedrine, that gave (after heating for 2 mins by 112C°) pink color with 2% ninhydrin in the chloroform fraction. Using GLC-MS technique, cathine and cathinone were also identified in chloroform fraction (ChF) with higher quantities in KD. Ethylacetate fraction (EaF) showed a trace amount whereas benzene fraction (BF) was free of them (data not show, ongoing chemical analysis).

Methanolic khat extracts and its fractionations contract ring preparations of rat aortas

Methanol extract of *Catha edulis* contracted ring preparations of rat aortas (Figure 1). Concentrations used of KL and KD were 250, 125 and 67.5 µg extract / 1 ml Krebs-Henseleit buffer (K-H). The corresponding induced contraction in comparison to phenylepherine induced contraction (control) were 80.2%, 57.3% , 26.4% and 78.4%, 60.8% , 24.6 % for KL and KD, respectively (N= 6). Removal of the endothelium did not influence the contraction. BF, ChF and EaF of KL and KD also contracted rat aorta rings (Figure 2). The contraction was reproducible with 2 micrograms of BF and EaF as well as 1 microgram of ChF per 1 ml K-H (Figure 2). Repetition of the dose (accumulation) of ethylacetate fraction (depending on the quantity added) produced slight contraction and then relaxation (N=8). The solvents, benzene, chloroform and ethylacetate did not show remarkable effect on the tissue.

Form of the induced contraction

The shape of contraction curve of EaF differed from those induced by chloroform and benzene fractionated extracts. The time needed to reach the plateau was shorter (4.3 ± 0.94 min) in EaF but longer in the two others (18± 2.2, 19.7± 0.94 min for BF and ChF, respectively, n= 8), (Figure 3).

Effect of nifedipine and prazosin on the contraction induced by khat fractionations

Nifedipine (10^{-6} M) non-significantly reduced the contraction induced by BF, ChF and EaF fractions, p= 0.77, 0.66, 0.67, and 0.77, 0.68, 0.13, for all fractions of KL and KD, respectively. On the other hand, pre-incubation of aortas with prazosin (10^{-6} M) reduced the contraction induced by all fractions (Figure 4a and 4b). The reduction in contraction was 81.9%, 63.1%, 71.8% with p= 0.23, 0.09, 0.15 for BF, ChF and EaF of KL, respectively. The reduction was significant in case of ChF (64.1%; p= 0.02) and EaF (73.5%; p= 0.04) of KD. Prazosin reduced BF (of KD) induced contraction insignificantly, 63.1%; p= 0.06.

Table 1: The total amount yielded by a solvent-solvent fractionation of the methanol extract (2 g) of *Catha edulis*

Used fractionation solvent	KL / Yield in mg	KD / Yield in mg	Status of the residue
Benzene	363	364	Black oily
Chloroform	43	34	Green amorphous
Ethyl acetate	434	563	Brown light crystals

A definite amount (2 g) of methanol extract of either khat light (KL) or khat dark (KD) was extracted with each of the solvents (see methods) and the residue was weighted.

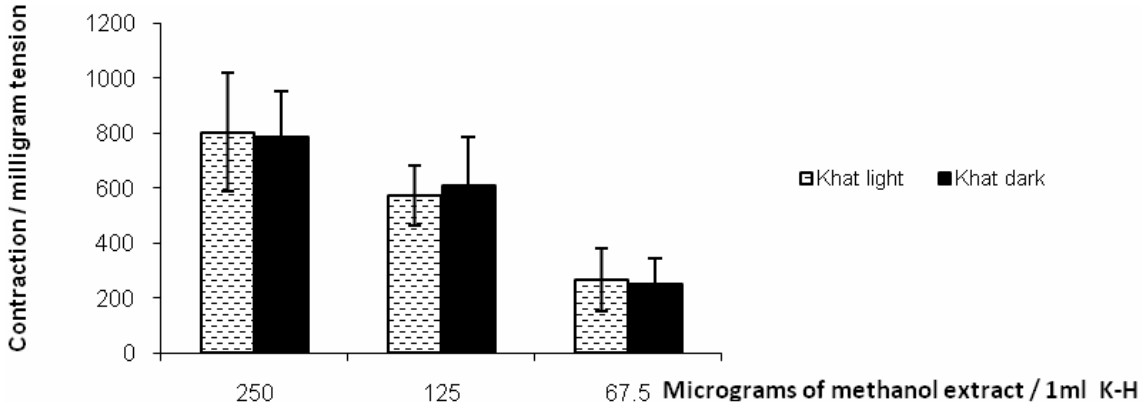


Figure 1: Contraction induced by methanol extract of *Catha edulis* in aorta ring preparations of rats Methanol extract, 250, 125 and 67.5 µg extract / 1 ml Krebs-Henseleit buffer (K-H), of KL and KD before fractionation was added to the ring bath. N= 6

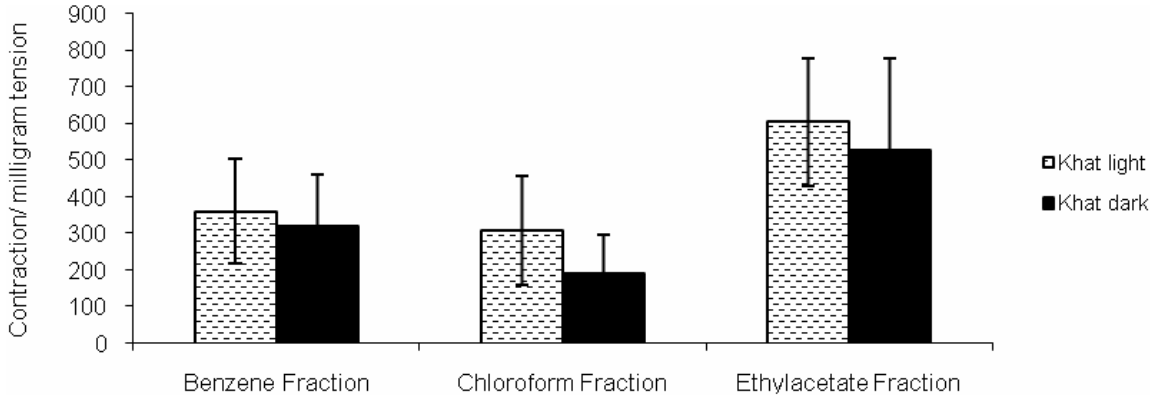


Figure 2: Contraction of aorta ring preparations of rats induced by khat fractionsBenzene, 2 µg/ 1 ml Krebs-Henseleit buffer (K-H), chloroform,1 µg/1ml K-H, and ethylacetate, 2 µg/ 1 ml K-H, fractions were added to the bath. The action lasted 30 mins. N=8

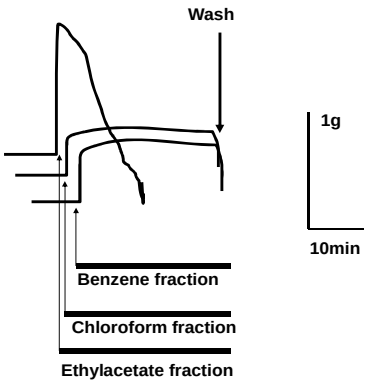
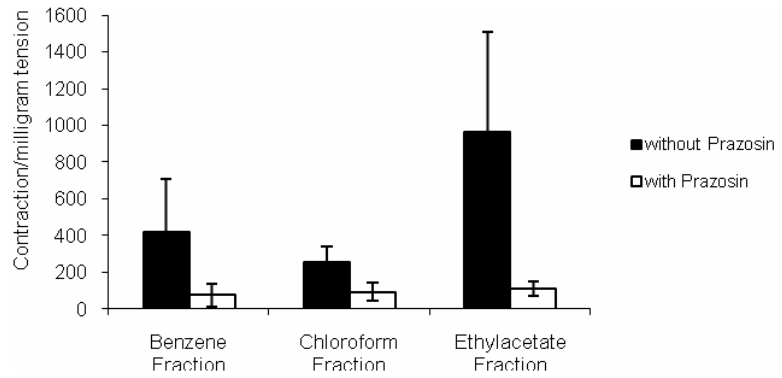


Figure 3 Original tracing showing khat effect in rat aorta rings At 0 minute the fraction was added and after 30 minutes washed.

4a.



4b.

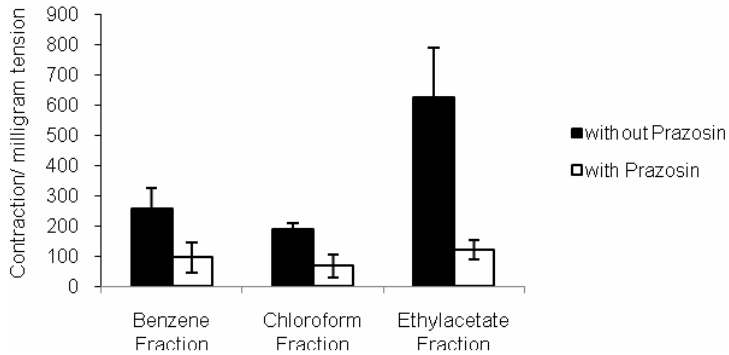


Figure 4: Prazosin reduced the contraction of rat aortas induced by khat . Prazosin (10^{-6} M) reduced the contraction of rat aortas induced by KL (a) and KD (b) fractions. The muscle was pre-incubated with Prazosin (10^{-6} M) for 20 mins. Then benzene (2 µg/ 1 ml K-H), chloroform (1 µg/1ml K-H) and ethylacetate (2 µg/ 1 ml K-H) fractions were added to the bath. Action lasted 30 mins. N= 5

Discussion

The study was carried out to investigate the effect of methanol extract and its fractions on rat aortas. Comparative differences between crimson and green leaves were included. The results demonstrate that methanol extracts of KL and KD contracted, depending on the added quantity, the rat aorta ring preparations. The contraction was not different when the endothelium was removed. Furthermore, fractions of the methanol extract showed contraction. It was reported that cathinone constricted guinea-pig aorta ring preparations and contraction induced by it reached its plateau by 25.4 ± 3.2 min in those preparations (Al-Motarreb and Broadley, 2004). ChF contracted our rat aortas. We detected cathinone in this fraction by using GC-SM. The contraction induced by ChF reached its plateau by 19.7± 0.94 min. Taking these together, it seems that our finding is in concord with that published by Al-Motarreb and Broadley (2004). Unexpectedly, BF contracted the rat aorta too. This finding is of interest since the fraction was devoid of active cathinone (under our conditions). Further chemical analysis and investigation are strongly recommended. In view of these findings, the raised question was to what extent the different substances present in the plant could contribute to the effects observed after khat chewing.

Interestingly we observed different shapes of contraction among the three fractionations within the same leaves (KL or KD), but they showed identical tendency in KL and KD. On the other hand, the solvents did not show any effect. The shape of contraction shown by EaF was suggestive of new components. Oxidation of these components gave a peak which gradually disappeared with increases in the concentration of the fraction. Using Molyneux method (Molyneux, 2004), that is based on color change of diphenylpicrylhydrazyl (free, stable radical; violet) into the reduced form diphenypicrylhydrazine, yellow, in the presence of anti-oxidizing components has given positive results (data not shown). Cumulative dose of the EaF produced relaxation, which needs to be further investigated.

In order to ensure good quality of the material, khat leaves were bought from well-known private farm in Gehaf Mountain, Aldhalea province, Yemen. They were sorted and prepared within two weeks from harvesting. The yields of methanol extract were almost equal to that found by Al-Hebshi et al (2005).

The reaction with ninhydrin suggested qualitatively the presence of phenylalkylamine character (cathinone group) in ChF, in which cathinone and cathine were (previously mentioned) identified by GC-MS. Under our conditions, high quantities of cathine and cathinone were obtained in KD (unpublished data) which is under investigation. We concluded that cathinone was present in chloroform fractions of both leave kinds with the highest amount in the chloroform fraction of crimson leaves.

The knowledge of khat pharmacology is increasing, but its interpretation remained fairly controversial in some aspects. Some of the peripheral effects may be over looked or simply escaped interpretation for various reasons, including poor understanding and investigation of ethnic khat chewers who started chewing khat since childhood or suffer some undefined masked diseases as well as absence of controlled studies of ethnic chewers. Therefore, elucidating the effects of this plant is still valuable. Early studies have shown that cathinone has amphetamine like actions and interacts with brain catecholamines by an indirect mechanism and, most probably, by affecting neurotransmitter release of the labile pool (Zeleger, et al., 1980), or by acting on the catecholaminergic synapses in the brain (Kalix, 1983 and 1984) to increase dopamine levels (Patel, 2000). In addition, cathinone increases blood pressure and heart rate through noradrenaline (norepinephrine) release from peripheral neurons similar to amphetamine (Kalix, 1992) increases in blood pressure and heart rate have been observed in human volunteers after chewing khat, which coincide with raised plasma cathinone concentrations (Widler, et al., 1994). More over, khat chewing is considered as a potential risk factor for acute myocardial infarction (Al-Motarreb, et al 2002; Alkadi, et al., 2002; Saha, and Dollery, 2006; Hassan, et al., 2005]. Recently, studies described the potency of vasoconstriction of cathinone in guinea pig aortas weaker than that of norepinephrine, and cathinone did not display indirect or direct sympathomimetic activity as well as tachyphylaxis (repetition of doses produced same previous contraction) and reaches plateau contraction after longer time (Al-Motarreb and Broadley, 2004). On the other hand, cathinone as well as amphetamine derivatives potentiate the actions of norepinephrine probably through competitive blockade of norepinephrine transporter (Clearly, et al., 2002; Connor, et al., 2002); therefore, cardiac morbidity was ascribed to it.

Nasher, et al. (1995) demonstrated that the overall urine flow rates were found to be significantly lower in khat users and this effect is probably mediated through stimulation of alpha-1 adrenoceptors in the bladder neck by the sympathomimetic alkaloid cathinone. These effects were abolished by the alpha 1 adrenoceptor blocker indoramine. So, to acquire some information about the mechanism of action of the fractions, we tried to block the contraction inducing pathways, calcium channels and sympathetic alpha-1 receptors. Therefore, nifedipine and prazosin were applied. Pretreatment with 10^{-6} M nifedipine insignificantly reduced the contractile activity of the fractionations, while 10^{-6} M prazosin significantly reduced the contraction of ChF and EaF of KD. However, prazosin appeared to reduce also the contracting activity of ChF of KL. There is no clear explanation for this finding, but the low quantity of cathinone and cathine in KL might be apart included in its analysis.

Significant contraction reduction of ChF of KD (p=0.02) by prazosin and the identified cathinone in KD suggested an alpha-sympathetic activity of the chloroform fraction. On the other hand, the contraction induced by BF of both KL and KD and its insignificant reduction by prazosin is interesting and a basis for further investigation. The data support the idea that khat may contain contracting substances other than cathinone to which untoward reactions (mainly cardiovascular) of chewing khat could be attributed.

In conclusion, fractionations of green and crimson Yemeni khat leaves contracted aortas of Lewis rats. Both leaves behave almost similarly. The contraction is due, in part, to cathinone and /or components other than cathinone. Contraction induced by chloroform fraction showed alpha-sympathetic activity.

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