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Theacrine, a purine alkaloid with anti-inflammatory and analgesic activities

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ABSTRACT

The anti-inflammatory and analgesic effects of theacrine (1, 3, 7, 9-tetramethyluric acid), a purine alkaloid which is abundantly present in *Camellia kucha*, were investigated. Xylene-induced ear edema, acetic acid-induced vascular permeability and λ-carrageenan-induced paw edema were used to investigate anti-inflammatory activity, and acetic acid-induced writhing and hot-plate tests were used to determine analgesic effect. Oral administration of theacrine (8–32 mg/kg) induced dose-related anti-inflammatory and analgesic effects. On the other hand, oral caffeine administration (8–32 mg/kg) did not show an inhibitory effect on the inhibition of inflammatory response or cause analgesia. Additionally, the result of the acute toxicity test showed that the LD_{50} of theacrine was 810.6 mg/kg (769.5–858.0 mg/kg). The data obtained suggest theacrine possessed analgesic and anti-inflammatory activities.

1. Introduction

Camellia kucha is a tea plant endemic to the wild woods of Yunnan, where it grows above 1000 m of altitude. *C. kucha* has been consumed as a tea for a long time [1], and has been found to contain a purine alkaloid (theacrine (TC) (Fig. 1) structurally different from caffeine, the predominant purine alkaloid found in traditionally cultivated tea plants [2–4]. Theacrine has shown interesting bioactivity. Previous pharmacological studies in laboratory animals have demonstrated that it acts on the central nervous system causing sedation, hypnoses and promoting memory function [5]. We have now investigated the activity of theacrine in animal models of inflammation and pain.

2. Material and methods

2.1. Plant material and crude extract preparation

Tea samples were collected in May 2008 from wild-grown trees of *C. kucha*. Fresh tea leaves were dried by oven at 80 °C.

2.2. Analysis of theacrine in C. kucha by HPLC

1 g sample was extracted in a 200 ml water bath for 30 min at 90 °C and shaken in 5 min intervals. The cooled sample was filtered through a 0.45 μm nylon filter and diluted to 1 l and analyzed directly by HPLC.

The samples were analyzed according to our previous work [6]. HPLC analytical condition: mobile phase A contained ortho-phosphoric acid (85%) and water (0.05:99.95, V: V); mobile phase B was acetonitrile (ACN). The gradient was as follows: 0–4 min, 2% B; 4–21 min, linear gradient from 2% B to 9% B; 21–32 min, linear gradient from 9% B to 23% B; 32–45 min, 23% B. Elution was performed at a solvent flow rate of 0.8 ml/min. The sample injection volume was 20 μl. Peak purity testing and detection were accomplished using Waters 2996 photodiode array detector (Waters, Mildford, U.S.A.).

2.3. The extraction of theacrine

50 g of dry leaves were extracted twice (30 min each) with 1000 ml of boiling water. After filtration, the filtrate was concentrated by evaporation under vacuum to 300 ml. The filtrate was treated with $Pb(OAc)_2 \cdot Pb(OH)_2$. After discarding the precipitate, the filtrate was extracted by 300 ml of

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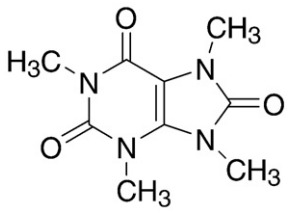

Fig. 1. The structure of theacrine (TC).

chloroform three times and evaporated chloroform to yield chloroform extract. The extract was subjected to chromatography over silica gel with petroleum ether and ethyl acetate (20:80) and further separated by preparative HPLC to yield 450 mg of theacrine. The structure was confirmed by spectral data (MS, NMR, element analysis). Peak purity testing was accomplished using HPLC with a Waters 2996 photodiode array detector.

2.4. Drug administration

All experiments were performed with male or female NIH mice, weighing 18–22 g, obtained from the Experimental Animal Center of Guangdong province, China. Animals were kept in plastic cages at 22 ± 2 °C with free access to food and water on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996).

Theacrine and test drugs were given orally to NIH mice after suspending in a mixture of distilled water and 0.5% sodium carboxymethyl cellulose (CMC-Na). The control mice received the same experimental handling as those of the test groups except that the drug treatment was replace with appropriate volumes of the dosing vehicle.

2.5. Ear edema induced by xylene

A previously described procedure was followed [7]. Briefly, each mouse was administered a single dose of a test drug or vehicle 1 h before the induction of ear edema by topical application of 20 μl xylene on both surfaces of the right ear. The left ear was used as a control. Mice were sacrificed by cervical dislocation 30 min after xylene application. Ear disks of 6.0 mm in diameter were punched out and weighed. The extent of edema was evaluated by the weight difference between the right and the left ear disks of the same animal.

2.6. λ-Carrageenan-induced mice paw edema

λ-Carrageenan-induced paw edema was carried out according to a previously described procedures [8], 20 μl of 2% λ-carrageenan suspension in normal saline was injected into the plantar side of right-hind paw of each mouse 1 h after oral administration of theacrine (8 mg/kg, 16 mg/kg and 32 mg/kg). Paw volume was measured with plethysmometer at 1, 2 h and 4 h after λ-carrageenan injection.

2.7. Vascular permeability

Vascular permeability tests were carried out according to a previously described method [9]. 30 min after the oral administration of theacrine (8 mg/kg, 16 mg/kg and 32 mg/kg), the mice received an i.v. injection of 1% Evan's blue solution (0.1 ml/10 g) to the tail. 10 min later an i.p. injection of 0.6% acetic acid solution in saline (0.1 ml/10 g) was given to each mouse to the increase of vascular permeability. 20 min after i.p. administration of acetic acid, the mice were euthanized. The inside of the abdominal cavity was washed with 10 ml of the physiological saline, which was collected and centrifuged at 3000 × *g* for 10 min. The concentration of Evan's blue in the fluid of the peritoneal cavity was measured by absorbance at 610 nm by UV–visible spectrophotometer. Indomethacin (10 mg/kg) was used as a control drug.

2.8. Acetic acid-induced writhing response

The abdominal constrictions resulting from intraperitoneal (i.p.) injection of 0.2 ml acetic acid (1.4%) consisting of the contraction of abdominal muscle together with a stretching of hind limbs, were carried out according to the previously described procedures [10]. The animals were pretreated with drugs. After 1 h, acetic acid was administered (i.p.). The number of writhing movements was counted for 20 min.

2.9. Thermally induced pain in mice

The hot-plate test was carried out according to the previously described method [11]. Animals were habituated twice to the hot-plate in advance. For testing, female mice were placed on a hot-plate maintained at 55 ± 0.5 °C. The time that elapsed until the occurrence of either a hind paw licking or a jump off the surface was recorded as the hot-plate latency. Mice with baseline latencies of <5 s or >30 s were eliminated from the study. After the determination of baseline response latencies, hot-plate latencies were redetermined at 30 min, 60 min, 90 min and 120 min after oral administration of theacrine (8 mg/kg, 16 mg/kg and 32 mg/kg).

2.10. Acute toxicity

The median lethal dose (LD_{50}) of theacrine was determined in the mice according to a modified method [12]. Mice fasted for 24 h were randomly divided into groups of ten mice per group of either sex. Graded doses of theacrine (450 mg/kg to 1000 mg/kg) were separately administered orally to the mice in each of the test groups. The mice in the test groups were then allowed free access to food and water and observed over a period of 14 days for signs of acute toxicity. The number of deaths caused by the theacrine within this period was recorded. Log–dose response plots are constructed for the plant extract, from which the median lethal dose (LD_{50}) of the theacrine was determined.

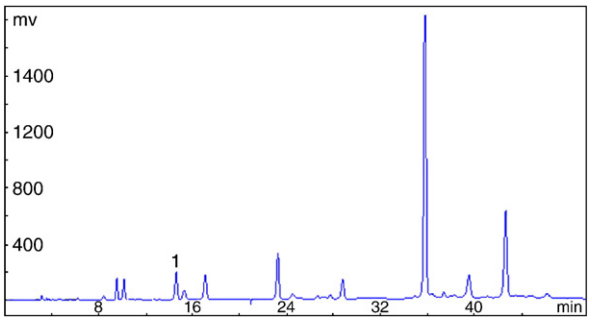

Fig. 2. The chromatogram of sample of *Camellia kucha*. 1. theacrine.

2.11. Statistical analysis

The results were expressed as mean ± S.D. and evaluated by one way ANOVA following by Student's *t*-test. $P<0.05$ was considered to be significant.

3. Results

Tea sample was analyzed by HPLC as illustrated in Fig. 2. The content of theacrine in *C. kucha* was 1.8% ± 0.05%. The identification of theacrine: Yield 50% m.p. 225–226 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.37 (s, 3H), 3.58 (s, 3H), 3.64 (s, 3H), 3.74 (s, 3H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 28.29 (q), 29.33 (q), 30.66 (q), 31.76 (q), 99.62 (s), 135.88 (s), 150.92 (s), 152.19 (s), 163.76 (s). MS (ESI): m/z (100%) = 225 (M + 1). Anal. Calcd for $C_9H_{12}N_4O_3$:C 48.37%, H 5.54%, O 21.05%, and N 25.04%. The purification of theacrine (99.6%) was verified using Waters 2996 photodiode array detector.

Oral administration of theacrine showed potent anti-inflammatory activities. Theacrine showed a significant effect to inhibit the ear edema induced by xylene in a dose-dependent manner ($P<0.05$) (Fig. 3). The inhibition percentages at each dose of theacrine (8 mg/kg, 16 mg/kg and 32 mg/kg) were 10.1%, 12.9% and 45.2%, respectively, compared with the control.

λ-Carrageenan-induced paw edema was also significantly reduced in a dose-dependent manner by treatment with theacrine 1, 2 and 4 h after injection of λ-carrageenan. A maximal inhibitory ratio of anti-edema effect of each dose (8 mg/kg, 16 mg/kg and 32 mg/kg) was observed at the 2 h ($P<0.05$) which was 14.1%, 16.6% and 20.1%, respectively, compared with 18.8% of indomethacin. Declining in activity was observed at 4 h which was 10.0%, 11.9%, 13.7%, respectively, compared with 21.6% of indomethacin (Table 1).

The anti-inflammatory activity of theacrine was investigated by the inhibition on vascular permeability at the same time. Theacrine at the dose of 16 mg/kg and 32 mg/kg inhibited the acetic acid-induced extravasation of Evan's blue dye by 10.6% and 18.9% as compared to the control ($P<0.05$). No significant effect was observed at the dose of 8 mg/kg (Table 2).

For the antinociceptive activity, the results showed that the extract significantly inhibited the writhing response induced by acetic acid and increased the pain threshold to hot-plate. As can be seen in Table 3, theacrine exhibited a significant analgesic effect against acetic acid- induced writhing response in mice ($P<0.05$) in a dose-dependent manner. Inhibitory ratios at the dose of 8 mg/kg, 16 mg/kg and 32 mg/kg were 24.5%, 30.3% and 34.1%, respectively. The inhibition ratio produced by a positive control indomethacin is 44.0%. Theacrine also significantly prolonged the latency to respond to acetic acid at 16 mg/kg and 32 g/kg doses with response values of 4.19 min and 4.38 min, respectively, compared with 3.32 min of the control. Results shown in Fig. 4 demonstrated that theacrine significantly increased hot-plate latency in a dose-dependent manner between 30 min and 120 min. The analgesic effect of theacrine in each group reached to the maximum at 90 min ($P<0.05$) which was 17.66 s, 20.90 s and 22.89 s, respectively, compared with the control of 10.99 s.

In addition, the acute toxicity of theacrine was evaluated in mice. Our results showed that the LD_{50} of theacrine was 810.6 mg/kg in mice (95% confidence interval 769.5–858.0 mg/ kg) which suggest that the doses we applied in the animal models are relatively safe since they are far below the LD_{50} of theacrine. Furthermore, the LD_{50} of theacrine is much lower when compared to the LD_{50} of the other familiar purine alkaloids which are abundantly present in *Camellia sinensis* including caffeine (LD_{50} 260 mg/kg in rats), theobromine (LD_{50} 600 mg/kg in mice) and theophylline (LD_{50} 300 mg/kg in rats) [13].

4. Discussion

The present study shows theacrine exhibits significantly anti-inflammatory and analgesic activities in a dose-dependent manner.

Xylene-induced ear oedema, λ-carrageenan induced paw edema and acetic acid-induced vascular permeability were used to investigate anti-inflammatory activity.

Xylene-induced ear edema is an acute inflammation model. Ear edema may involve inflammatory mediators

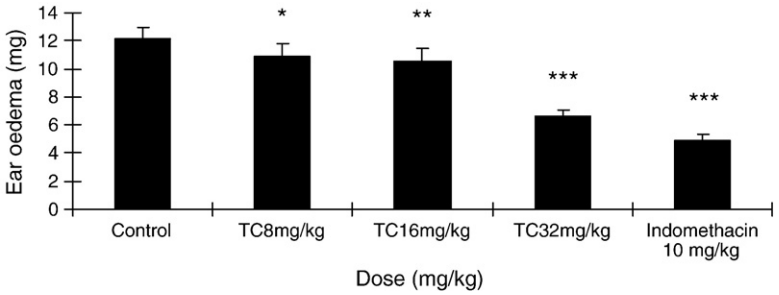

Fig. 3. Effects of TC on xylene-induced ear oedema. Results are expressed as mean ± S.D. (n = 8). *P<0.05, **P<0.01 and ***P<0.001 compared with control.

Table 1
Effects of TC on λ-carrageenan-induced paw edema in mice.

Group	Dose (mg/kg)	Paw swelling (ml)			Inhibition (%)		
		1 h	2 h	4 h	1 h	2 h	4 h
Control	–	0.288 ± 0.019	0.309 ± 0.027	0.328 ± 0.043	–	–	–
TC	8	0.261 ± 0.033*	0.269 ± 0.019**	0.295 ± 0.015*	9.3	14.1	10.0
	16	0.255 ± 0.016*	0.261 ± 0.024**	0.289 ± 0.028*	11.5	16.6	11.9
	32	0.243 ± 0.029**	0.250 ± 0.044**	0.283 ± 0.031**	15.6	20.1	13.7
Indomethacin	10	0.244 ± 0.046**	0.251 ± 0.014**	0.257 ± 0.052**	15.3	18.8	21.6

Results are expressed as mean ± S.D. ($n = 8$). *$P<0.05$ and **$P<0.01$ compared with control.

such as histamine, serotonin, and bradykinin. These mediators can induce ear edema by promoting vasodilatation and increasing vascular permeability [14]. Our results show that theacrine can significantly inhibit the formation of xylene-induced ear edema. Theacrine may interfere with the actions of the above mediators to produce the anti-inflammation effect.

λ-Carrageenan-induced hind paw edema in mice is a biphasic event. The early phase (90–180 min) of the inflammation is due to the release of histamine, serotonin and similar substances, and the later phase (270–360 min) is associated with the activation of kinin-like substances [15]. The results of λ-carrageenan experiment indicated that theacrine mainly functions on the first phase of inflammation presumably by interacting with histamine and serotonins and other similar mediators.

In addition, the anti-inflammatory activity of theacrine is also shown by the results of the vascular permeability induced by acetic acid. The vascular permeability induced by acetic acid causes an increase in peritoneal fluids of prostaglandin, serotonin, and histamine. This leads to a dilation of the capillary vessels and the increase in vascular permeability. As a consequence, fluid and plasma proteins are extravasated, and edema forms [16]. Theacrine shows significant anti-inflammatory effects in vascular permeability, and thus it may have a membrane stabilizing effect that reduces capillary permeability.

In the above acute inflammatory models, theacrine shows anti-inflammatory activity. These data suggest that theacrine has an anti-inflammatory property probably acting through the inhibition of the inflammatory mediators of the acute phase of inflammation.

The results obtained in the analgesic test experiments showed that theacrine significantly increased the pain threshold on the hot-plate and inhibited the writhing response induced by acetic acid, which suggests that theacrine possesses both centrally and peripherally mediated analgesic properties. The central analgesic action may be mediated via inhibition of central pain receptors, while the peripheral analgesic effect may be mediated through inhibition of cyclooxygenase and/or lipoxygenase and other inflammatory mediators. This hypothesis is postulated by the previous reports that hot-plate test and acetic acid writhing methods are useful techniques for the evaluation of centrally and peripherally acting analgesic drugs, respectively [17–19].

On the other hand, theacrine also shows a certain degree of toxicity as other alkaloids, while it's still much safer when compared to the other familiar purine alkaloids abundantly present in *C. sinensis*.

In our models, we did not observe individual anti-inflammatory and analgesic effects of caffeine at the range of doses tested (8–32 mg/kg) (data not show). These results are in accordance with previously published reports [20–22] where only doses up to 50 mg/kg were effective in relieving inflammation and nociception, respectively; but doses at high level are near the threshold of toxicity in animals and have questionable relevance for their use in humans.

Theacrine displayed analgesic and anti-inflammatory activities which may be related to distinctive structural differences from the predominant purine alkaloid in traditionally cultivated tea plants. The previous studies by Xu et al showed that theacrine acts on the central nervous system causing sedation, hypnoses, an effect completely opposite to caffeine, which suggests that theacrine may exert its effects as agonists of GABA receptor or increase the available amount of

Table 2
Effects of TC on acetic acid-induced vascular permeability in mice.

Group	Dose (mg/kg)	OD	Inhibition (%)
Control	–	0.083 ± 0.079	–
TC	8	0.080 ± 0.065	3.0%
	16	0.074 ± 0.058**	10.6%
	32	0.067 ± 0.038***	18.9%
Indomethacin	10	0.052 ± 0.022***	37.3%

Results are expressed as mean ± S.D. ($n = 8$). **$P<0.01$ and *** $P < 0.001$ compared with control.

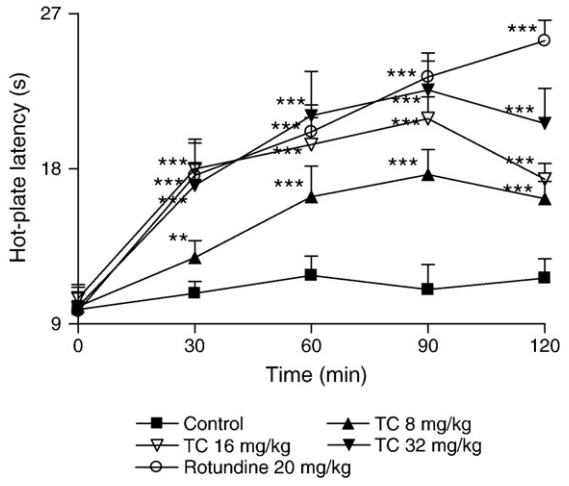

Fig. 4. Effects of TC on hot-plate tests. Results are expressed as mean ± S.D. (n = 8). **P<0.01 and *** P<0.001 compared with control.

Table 3
Effects of TC on acetic acid-induced writhing response in mice.

Group	Dose (mg/kg)	Latency (min)	Number of writhing	Inhibitory ratio (%)
Control	–	3.32 ± 0.39	30.83 ± 4.66	–
TC	8	3.71 ± 0.32	23.28 ± 3.60*	24.5%
	16	4.19 ± 0.59**	21.50 ± 5.31*	30.3%
	32	4.38 ± 0.47**	20.31 ± 2.52*	34.1%
Indomethacin	10	4.25 ± 0.43**	17.25 ± 2.74**	44.0%

Results are expressed as mean ± S.D. ($n = 8$). *$P<0.05$ and **$P<0.01$ compared with control.

GABA [23]. It may be another possibility to explain the antinociceptive effect of the theacrine on centrally mediated analgesic, since GABA is known to induce nociception [24]. Further investigation is required to elucidate the mechanisms underlying these effects.

Acknowledgments

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