ACUTE TOXICITY AND ANALGESIC EFFECTS OF KHOP BAO AN CAPSULES IN EXPERIMENTAL ANIMALS

Nguyen Thi Minh Thu[1], Pham Thanh Tung[1], Trinh Dinh Nhat Duy[2], Nguyen Thi Anh Hong[1], Nguyen Binh Minh[3]

[1]*Vietnam University of Traditional Medicine*

[2]*Quy Nhon Traditional Medicine and Rehabilitation Hospital*

[3]*National Hospital of Tropical Diseases*

ABSTRACT

Objective: *To evaluate the acute oral toxicity and analgesic effects of Khop Bao An (KBA) capsules in mice.*

Subjects and methods: *KBA's acute toxicity and analgesic effects were assessed according to Ministry of Health guidelines and using hot plate and acetic acid-induced writhing tests.*

Results: *At the highest dose that mice could ingest, 26.42g of mixed dry extract per kg, KBA did not cause deaths. All mice remained healthy and showed significant weight gain on day 7 compared to that on day 0 as well as had normal visceral organs under macroscopic examination. In addition, at doses of 989.28 and 2,967.84 mg of mixed dry extract/kg/day for 7 consecutive days, mice were able to notably prolong the duration of pain tolerance to heat stimulation compared to those before treatment and the control group. Also, the numbers of mice's writhing episodes at each 5-minute interval within 30 minutes after injecting acetic acid were remarkably reduced, and the times to the first writhing episode were longer than those of the control group.*

Conclusion: *KBA did not cause acute toxicity in mice and had both significant central and peripheral analgesic effects at the tested doses.*

Keywords: *Khop Bao An capsule, acute oral toxicity, analgesic effects.*

INTRODUCTION

Osteoarthritis is a common disease among middle-aged people, significantly impacting their daily lives and quality of life. Currently, the main medicinal chemistry products used to treat this disease are glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs). While highly effective in improving symptoms, these drugs also carry a number of health risks related to cardiovascular, gastrointestinal, hematological systems, liver and kidney functions, as well as osteoporosis [1],[2]. Therefore, the development of herbal-based drugs with impactful efficacy and reduced side effects is a highly prioritized trend among researchers nowadays.

Khop Bao An (KBA) capsules have been formulated from nine medicinal herbs. This is a combination used according to folk experiences to treat osteoarthritis; however, there has been no systematic scientific research to prove its effectiveness. Several studies have been conducted to evaluate the pharmacological effects of each herb in the above combined formulation when used individually, such as analgesic [3],[4],[5], anti-inflammatory [6],[7],[8], antioxidant [6],[9], antibacterial [5],[7],[9], and uric acid-lowering effects [8]. Besides, there is very little information on the acute toxicity of single-ingredient medicinal herbs except for the studies by Muhua Yuan et al. (2019) and Soo-Wang Hyun et al. (2021) on the acute toxicities of *Rhizoma Smilacis glabrae* in mice and *Achyranthis Radix* extract in rats, respectively [10],[11]. The question is how the toxicity and analgesic/anti-inflammatory effects of the combination will manifest when these medicinal herbs are combined in the same formulation.

Although it has been traditionally used to treat arthritis in humans, to date, no scientific studies have been published on the safety and analgesic or anti-inflammatory effects of the combination of the nine herbs mentioned above. Thus, to contribute to the development of this combination drug for the treatment of osteoarthritis, we conducted this study to evaluate the acute oral toxicity and analgesic effects of KBA in experimental animals.

MATERIALS AND METHODS

Test sample

Khop Bao An capsules (KBA) were manufactured by Bach Thao Duoc Factory Joint Stock Company according to the company's standards (TCKT.TP.03). Each capsule contained 458 mg of mixed dry extract equivalent to 4.58 g of dried medicinal herbs, consisting of *Caulis Tinosporae sinensis* 1.0g, *Rhizoma Drynariae* 0.5g, *Radix Achyranthes bidentatae* 0.5g, *Radix Dipsaci* 0.5g, *Radix Polygoni cuspidati* 0.5g, *Caulis Spatholobi suberecti* 0.5g, *Rhizoma Smilacis glabrae* 0.5g, *Cortex Oroxyli indici* 0.5g and *Ramulus Cinnamomi* 0.08g. Thus, the ratio of dry medicinal herbs to extract was 10:1. All nine medicinal herbs had met the standards of the Vietnamese Pharmacopoeia V [12] before being processed into mixed dry extracts and formulated into capsules. The test sample was diluted with distilled water to prepare suspensions of varying concentrations for the experiments.

Corresponding author: Nguyen Thi Minh Thu

Phone: (+84)912750167

E-mail: minhthunimpe@gmail.com

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KBA is intended to treat pain and inflammation of osteoarthritis with an average clinical dose of 9 capsules/day, equivalent to 4.122g of dry extract/day/person. For an average person weighing 50kg, the expected dose would be 0.08244g of dry extract/kg/day, equivalent to 989.28 mg of mixed dry extract/kg/day in mice.

Experimental animals

Swiss albino mice, provided by the National Institute of Hygiene and Epidemiology, met the following criteria: 4-5 weeks old, weighing 20 ± 2g, mature, healthy, and indistinguishable by sex. Female mice were not pregnant and had never reproduced. The mice were kept in stable conditions for 3 days before the study with temperatures of 22-25°C, humidity of 50-60%, and a 12/12-hour light-dark cycle, with the light cycle from 6:30 AM to 6:30 PM.

The research was conducted at the Vietnam University of Traditional Medicine.

Drugs and chemicals

Diclofenac sodium 75 mg tablets (Uphace), batch 010424, expiry date 04/2027, VD-23083-15. Codeine phosphate (National Institute of Drug Quality Control), batch 0036721.04, expiry date 4/2026. Acetic acid solution, content ≥ 96% (Duc Giang Chemical Group Joint Stock Company), batch 01022024, 31:2008/HCDG.

Time and location

This study was conducted between April and June 2024 at Vietnam University of Traditional Medicine.

Methods

Acute toxicity:

This experiment was conducted in accordance with the guidelines of the Ministry of Health [13]. After 3 days of stable rearing, mice were marked, weighed, and randomly divided into groups of 10. Before administration, the mice were fasted overnight (16 hours). Next, they were given the test sample suspension in a single day by directly inserting the test sample into their stomachs through a blunt needle with progressively increasing doses in groups of 12.33, 15.85, 19.37, 22.90 and 26.42 g of mixed dry extract. Two hours after the last dose, the mice were allowed to eat and drink normally. Then, all mice were monitored for 1 week for their behavior including walking, climbing, respiration, neurological symptoms, eating, fur condition, feces, urine, signs of toxicity, and the number of dead mice if any. On day 7, all tested mice were dissected for gross examination of organs comprising their hearts, livers, kidneys, lungs, bladders, and intestines. If abnormalities were found, histopathological microsurgery was performed. The LD50 value (if available) was determined using linear regression analysis on the Probit program [13].

Analgesic effects:

Hot plate test:

Before administering the test sample, each mouse was placed on a heated tray maintained at a stable temperature of 55°C using a thermostat. The time from placing the mouse on the heated tray until it showed signs of raising its hind legs or licking its feet was recorded. Mice that reacted too quickly (before 8 seconds) or too slowly (after 30 seconds) were discarded. Next, the mice were divided into four groups of 10 mice each: (i) Group 1 (the control) where mice were given distilled water in a volume of 0.1 ml/10 g of body weight; (ii) Group 2: Mice orally received codeine phosphate 20 mg/kg/day; (iii) (iv) Groups 3 and 4: Mice were taken KBA at doses of 989.28 (equivalent to the therapeutic dose in humans) and 2,967.84 (three times the clinical equivalent dose) mg of mixed dry extract /kg/day, respectively, for 7 consecutive days. All mice were treated once daily in the morning for 7 consecutive days. On the last day, 1 and 2 hours after being taken the test sample, they were placed on the hot plate and their reaction times were recorded. Comparisons of their response times to heat were performed [14].

Acetic acid–induced writhing test:

This test was performed on mice in four groups of 10 each. In which, mice in the group 1 were given distilled water at a volume of 10 ml/kg body weight. Mice in group 2 were given Diclofenac sodium 24 mg/kg/day, while those in groups 3 and 4 were given KBA at dose regimens of 989.28 and 2,967.84 mg of mixed dry extract/kg/day, respectively, for seven consecutive days.

On the seventh day (D7), 60 minutes after administration, all mice were induced with writhing by injecting a 0.6% acetic acid solution into their peritoneal cavities at a dose of 0.1 ml/10 grams body weight. The onset times of mice's first pain episodes and their numbers of writhing episodes every 5-minutes from the time of peritoneal injection until 30 minutes after that were recorded. The results among the study groups were compared, and % inhibition of mice's writhing was calculated using the following formula: A% = (Dc-Dt)/Dc ×100. In which, A% is the percentage reduction in the number of writhing episodes in mice in the test group or reference drug group while Dc and Dt are the number of writhing episodes in the physiological control and the test group, respectively [15].

Data processing

Data are expressed as means ± standard deviations (Mean ± SD). They were processed using SPSS 22.0 software according to medical statistics methods, employing Student's t-test, Avant-Apres test (Before-After Test), and One-way ANOVA to compare data before, during, and after the trial, as well as the treatment groups with the control group. A p-value < 0.05 was considered statistically significant for differences between data.

Research ethics

This study adhered to the guidelines of the Ministry of Health in Decision 141 [13]. The number of animals used was sufficient for statistical purposes and there was no animal abuse. Animals that died during or survived after

testing were disposed of according to regulations. The research was conducted solely for the purpose of developing herbal-derived drugs and had no other conflicts of interest.

RESULTS

After administering KBA, all mice remained active, consumed food and water normally, showed no respiratory or central nervous system abnormalities, had smooth fur, dry molding feces, and pale-yellow urine. Moreover, no mortality occurred and mice's internal organs on day 7 were observed to be normal.

Table 1. Conditions of mice after oral administration of KBA

Group	Oral dose (g of dried herbal material/kg)	Oral dose (g of mixed dry extract/ kg)	Mortality rate (%)	Mice's weight (g)		p (D0-D7)
				Before KBA (D0)	After KBA (D7)	
1	123.3	12.33	0	20.79 ± 1.46	24.92 ± 0.65	< 0.001
2	158.5	15.85	0	20.78 ± 1.84	25.14 ± 0.76	
3	193.7	19.37	0	20.33 ± 1.36	24.57 ± 0.45	
4	229.0	22.90	0	20.58 ± 1.39	24.85 ± 0.82	
5	264.2	26.42	0	20.37 ± 1.77	24.74 ± 0.70	
p				> 0.05	> 0.05	

At all tested doses, KBA did not cause death in mice. Mice weights on day 7 increased remarkably compared to those on D0 ($p<0.001$). However, the weight of mice across the different groups at D7 did not differ notably ($p > 0.05$).

Table 2. Mice's responses to heat stimuli before and after KBA administration

Group (n = 10)	Mice's response times to thermal stimuli (seconds, means ± SD)			p (D0-D7.1), p (D0-D7.2)
	Before KBA (D0)	After KBA 1 hour (D7.1)	After KBA 2 hours (D7.2)	
1	13.22 ± 2.41	11.68 ± 2.96	11.67 ± 3.49	> 0.05
2	13.35 ± 2.78	22.33 ± 7.08	24.95 ± 2.07	< 0.001
3	12.32 ± 4.01	20.41 ± 4.81	21.98 ± 3.70	< 0.001
4	12.19 ± 2.51	17.3 ± 2.65	20.48 ± 4.55	< 0.001
p (1-2), (1-3), (1-4)	> 0.05	< 0.001	< 0.001	

Before the experiment (D0), mice in all four groups had similar heat response times ($p>0.05$). The heat response times of mice in the control group before and after being drunk distilled water did not differ significantly ($p > 0.05$). Meanwhile, mice in groups 2, 3, and 4 all showed notable increases in heat response times on day 7 compared to those before administration ($p<0.001$). Specifically, mice in the codeine phosphate group had the longest heat response time after 1 and 2 hours of treatment, followed by the group treated with KBA 989.28 mg/kg/day. Conversely, the group treated with the high dose of KBA (2,967.84 mg/kg/day) had shorter heat response times compared to those of the one treated with the lower dose of KBA; however, the difference was not statistically significant. In addition, the reaction times to heat in groups 2, 3, and 4 on day 7 differed not remarkably ($p>0.05$) but were statistically significantly higher than that of the control group ($p<0.001$).

After acetic acid injection, all mice exhibited abdominal muscle spasms, abdominal sagging to the cage floor, and elongated bodies, while some cried out in pain.

Table 3. Times of pain onset and number of writhing episodes in mice

Group (n = 10)	Number of writhing episodes in mice (mean ± SD)						Time of pain onset (minutes)
	0-5′	5-10′	10-15′	15-20′	20-25′	25-30′	
1	2.9 ± 0.99	13.6 ± 3.10	17.7 ± 4.24	13.2 ± 4.32	10.6 ± 3.59	5.0 ± 1.15	3.042 ± 0.110
2	0.8 ± 0.79	5.4 ± 2.59	8.0 ± 3.16	4.6 ± 3.13	2.1 ± 1.45	1.8 ± 0.79	5.004 ± 0.539
3	1.6 ± 0.70*	6.1 ± 2.13	11.2 ± 4.78	4.7 ± 1.83	2.6 ± 1.26	2.1 ± 0.87	4.658 ± 0.212
4	1.2 ± 0.79	5.5 ± 2.37	9.4 ± 4.62	3.5 ± 1.58	2.2 ± 1.36	2.0 ± 0.67	4.790 ± 0.173

*(p (1-2), p (1-3), p (1-4) < 0.001 and * < 0.01 compared to the control group)*

The number of mice's spasm episodes in groups 2, 3, and 4 after acetic acid injections was statistically significantly lower compared to that of the control group ($p<0.001$ and <0.01). KBA at the higher dose of 2,967.84 mg/kg/day tended to have a better analgesic effect than the dose of 989.28 mg/kg/day, with fewer spasm episodes at each time point; nevertheless, these differences were not remarkable ($p>0.05$). Also, mice orally treated with

diclofenac 24 mg/kg/day had fewer spasm episodes at each time point compared to those treated with KBA, except for the 15–20-minute period after acetic acid injection. However, the differences among groups 2, 3, and 4 were not statistically significant ($p>0.05$). On the contrary, in the first 5 minutes after injection, the number of spasm episodes in the diclofenac-treated group was significantly lower than that of the KBA group treated with a dose of 989.28 mg/kg/day ($p<0.05$). Apart from that, the time to onset of the first pain episode was remarkably lower in the control group compared to those of the others ($p<0.001$). Mice in the diclofenac-treated group had the latest onset of pain, followed by the high-dose KBA group, and then the low-dose one. However, the time to onset of pain among groups 2, 3, and 4 did not differ significantly ($p>0.05$).

Table 4. Percentages of reduction in the number of spasm episodes in mice

Group (n = 10)	Percentages of reduction in mice's spasm pain compared to the control group (%)					
	0-5′	5-10′	10-15′	15-20′	20-25′	25-30′
2	72.41	60.29	54.80	65.15	80.19	64.00
3	44.83	55.15	36.72	64.39	75.47	58.00
4	58.62	59.56	46.89	73.48	79.25	60.00

Compared to the control group, mice treated with KBA and diclofenac revealed significant reductions in the number of spasmodic pain episodes at each 5-minute interval after acetic acid injection.

DISCUSSION

Acute toxicity testing is usually the first test performed when evaluating drug's safety to classify its toxicity level. At all tested oral doses of 12.33, 15.85, 19.37, 22.90, and 26.42 g of mixed dry extract per kg, the mice remained healthy, gained significantly more weight than before administration, and none died. These demonstrate that, even at the highest dose tested (26.42 g of mixed dry extract/kg, 26.71 times higher than the equivalent dose used in humans), KBA capsules remained safe for mice. To date, no studies have evaluated the acute toxicity of a combination of nine herbal ingredients as in KBA capsules. Very few studies have evaluated the acute toxicity of each component. For example, Muhua Yuan et al. (2019) found that *Smilacis glabrae Rhizoma* did not cause acute oral toxicity in mice at a dose of 5.0 g/kg [10]. Subsequently, in 2021, Soo-Wang Hyun et al. determined that the extract of *Radix Achyranthis bidentatae* did not cause acute toxicity in rats at single doses of 0.5, 1.0 or 2.0 g/kg [11]. Our research results are consistent with those of the two authors mentioned above. Both medicinal herbs were present in equal amounts in the KBA capsules, at 28.8428 g of dried medicinal herbs each, within a 26.42 g of mixed dry extract, the highest dose that 1 kg of mice could tolerate in one day. This demonstrates that KBA capsules were well tolerated at this dose. On the other hand, the dosage of the medicinal herbs in our experiment was much higher than in the two previous experiments, possibly due to the use of a highly concentrated extract and multiple administrations in a single day. The purpose of these was to assess the maximum tolerability of mice in the short term. In brief, KBA did not cause acute toxicity at the tested dose, and the LD50 dose has not yet been determined.

The hot plate test applies heat stimuli to the hind paws, which are considered to integrate supraspinal pathways, as rats with spinal transection do not withdraw the hind limbs in the test. In the conventional hot plate test, an unrestrained mouse or rat is placed on a metal surface maintained at a constant temperature, usually between 50 and 55°C, and the response latency, which is the time taken to observe a nociceptive behavior, is recorded. Nociceptive behaviors comprise forepaw or hind paw withdrawal or licking, stamping, leaning posture and jumping. Although forepaw withdrawal often occurs first, hind paw withdrawal or licking is considered to be a more reliable indicator of nociception, as the forepaws are frequently used in grooming and exploration and are not consistently in contact with the metal [14]. Given the above data, the hot plate test aims to evaluate the central analgesic effect of KBA. Thus, we chose codeine phosphate as the reference drug. In this test, at two oral doses of 989.28 and 2,967.84 mg of mixed dry extract/kg/day for 7 consecutive days, KBA significantly prolonged the duration of heat sensitivity in mice compared to those before administration and those of the control group. At the dose equivalent to the clinical therapeutic dose, KBA appeared to prolong the heat response time in mice better than at the dose three times higher, but the difference was not statistically significant. Further studies are needed to evaluate the correlation between responses and doses. Although these analgesic effects were lower than that of codeine phosphate 20 mg/kg, the differences among the three groups were not remarkable. Therefore, it can be concluded that, KBA has central analgesic effects similar to those of codeine phosphate at 20 mg/kg/day.

In addition, KBA's peripheral analgesic activities were also evaluated using the writhing test. This method uses chemicals to induce peripheral pain by injecting pain-inducing substances such as acetic acid or phenyl quinone into mice. Analgesic activities of the test sample are inferred from the decrease in the frequency of writhing in the animals. Writhing episodes are due to

profound pain of endogenous nature which recur for a prolonged period of time. Due to their irritant nature, these principles are also prone to inducing lesions. Writhing episode is an overt response to the intense pain induced by irritant principles via nociceptors characterized by episodes of retraction of the abdomen and stretching of hind limbs. The signals transmitted to the central nervous system in response to pain due to irritation, cause release of mediators such as prostaglandins which contribute to the increased sensitivity to nociceptors [15]. For this peripheral analgesia trial, a reference drug from the NSAID class such as diclofenac sodium is a reasonable choice. With the same two dosage regimens as above, KBA significantly reduced the number of writhing episodes in mice compared to the control group at each study time point. Simultaneously, the times to onset of pain in the groups treated with KBA and diclofenac were significantly longer than that of the control group. Additionally, the rates of reduction in the number of writhing episodes reached highest values after 25 minutes in the two groups receiving KBA, which were not significantly lower than the group of mice receiving diclofenac. These show that the pain protection rates of KBA are very powerful. Even though KBA showed lower analgesic activity than sodium diclofenac, the differences among three groups were not notable. In short, KBA had effective peripheral analgesic effects comparable to diclofenac 24 mg/kg/day.

The analgesic effects described above are consistent with the chemical composition reports of the medicinal components in KBA, with direct or indirect mechanisms of action. For example, flavonoids like rutin, quercetin, and isorhamnetin in *Caulis Tinosporae sinensis* inhibit the production of inflammatory cytokines and downregulate the MAPK signaling pathway, thus reducing inflammation and indirectly reducing pain [3]. Naringin, a flavonoid found in *Rhizoma Drynariae*, has analgesic and anti-inflammatory effects through the inhibition of inflammatory mediators such as COX-2. This compound helps reduce pain, inflammation, and supports wound healing due to its strong antioxidant properties. Astilbin, a flavonoid compound isolated from the rhizome of *Smilax glabra* Roxb, reduced joint damage in the hind legs of mice at a daily oral dose of 5.3 mg/kg/day. Accordingly, astilbin demonstrated a notable inhibitory effect on TNF-α, IL-1β, and IL-6 mRNA expression and significantly reduced serum cytokine levels in mice [10]. Chrysin and baicalein, the two main active ingredients of *Oroxylum indicum*, also showed good anti-inflammatory and analgesic effects in rats, with baicalein showing better analgesic effects than chrysin [16].

This is the first study to evaluate analgesic effects of a combination drug containing nine medicinal herbs like KBA capsules. Previously, several studies had determined the analgesic effects of some individual medicinal herbs when used separately. For example, the ethanolic extract of *Tinospora sinensis* leaves exhibited good analgesic effects in mice in two models of tail flick and acetic acid-induced writhing tests at 500 mg/kg [3]. In 1997, Lu T. et al. found that among processed products of *Achyranthes bidentata*, the ethanol extract showed the strongest and longest-lasting analgesic effect compared to other extracts [4],[17]. Another study on the analgesic effect of the n-butanol extract of *Oroxylum indicum* 100 mg/kg in mice with acetic acid-induced spasm found that oral administration of the test sample significantly prolonged mice's reaction times and notably reduced the number of seizures by 75.93% compared to aspirin (87.05%) [5].

Our results regarding the combination drug are consistent with those of the aforementioned authors when evaluating the analgesic effects of the individual components. These suggest that KBA exhibits impactful central and peripheral analgesic effects at the tested doses.

CONCLUSION

KBA capsules demonstrated a high safety profile, with no mortality or physiological abnormalities observed at a dose up to 26.42 g of mixed dry extract/kg, equal to 264.2 g of dried herbal material/kg. Regarding analgesic effects, oral administration of KBA (989.28 and 2,967.84 mg of mixed dry extract/kg) significantly enhanced mice's thermal tolerance in the hot-plate test and exhibited potent analgesic effects by reducing acetic acid-induced writhing and delaying pain onset. Although the high-dose group showed a trend toward superior pain relief, the difference between the two tested doses was not statistically significant.

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