การประเมินความปลอดภัยระดับพรีคลินิกของตำรับยากวาดสมานลิ้นขาวละออ สำหรับการสมานแผลภายหลังการให้ทางปากเป็นเวลา 28 วัน ในหนูแรทสายพันธุ์ Wistar

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บทคัดย่อ

ข้อมูลความเป็นพิษจากการได้รับยาซ้ำมีความสำคัญต่อการประเมินความปลอดภัยต่อร่างกายของตำรับสมุนไพรที่มีสรรพคุณสมานแผล ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินความเป็นพิษจากการได้รับตำรับยากวาดสมานลิ้นขาวละออ (KLO) ทางปากซ้ำเป็นระยะเวลา 28 วัน ในหนูแรทสายพันธุ์ Wistar วิธีการทดลองดัดแปลงจาก OECD Test Guideline 407 โดยให้ KLO ทางปากด้วยวิธี gavage วันละ 1 ครั้ง ที่ขนาด 0.1, 1 และ 5 มก./กก. น้ำหนักตัว/วัน และประเมินการตาย อาการแสดงทางคลินิก พฤติกรรม น้ำหนักตัว ปริมาณอาหารและน้ำ ค่าน้ำหนักสัมพัทธ์ของอวัยวะ การตรวจพยาธิสภาพด้วยตาเปล่า และจุลพยาธิวิทยา ผลการศึกษาไม่พบการตาย อาการแสดงทางคลินิกที่สัมพันธ์กับการได้รับตำรับ ความผิดปกติทางพฤติกรรม การเปลี่ยนแปลงน้ำหนักตัว ปริมาณอาหารหรือน้ำ หรือค่าน้ำหนักสัมพัทธ์ของอวัยวะในหนูที่ได้รับ KLO การตรวจพยาธิสภาพด้วยตาเปล่าและจุลพยาธิวิทยาไม่พบรอยโรคที่สัมพันธ์กับการได้รับตำรับ พบเพียงการเปลี่ยนแปลงทางโลหิตวิทยาบางค่าในหนูเพศผู้กลุ่มที่ได้รับ KLO ขนาดสูง ได้แก่ ค่า mean corpuscular volume และร้อยละของ granulocyte ลดลง อย่างไรก็ตาม การเปลี่ยนแปลงดังกล่าวไม่แสดงความสัมพันธ์กับขนาดยาอย่างชัดเจน และไม่มีหลักฐานสนับสนุนจากอาการทางคลินิก น้ำหนักอวัยวะ พยาธิสภาพด้วยตาเปล่า หรือจุลพยาธิวิทยา โดยสรุป การให้ KLO ซ้ำทางปากไม่ก่อให้เกิดความเป็นพิษต่อระบบของร่างกายที่ไม่พึงประสงค์ในระดับที่ตรวจพบได้ ค่า NOAEL ของ KLO เท่ากับ 5 มก./กก. น้ำหนักตัว/วัน ซึ่งเป็นขนาดสูงสุดที่ใช้ทดสอบในการศึกษานี้

คำสำคัญ: ยากวาดสมานลิ้นขาวละออ ยาสมานแผล ความเป็นพิษกึ่งเฉียบพลัน ค่า NOAEL

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Preclinical Safety Evaluation of the Khaolaor Ya-Kwad-Samarn-Lin Wound-Healing Formulation Following 28-Day Oral Administration in Wistar Rats

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Abstract

Toxicity data following repeated administration are essential for evaluating the systemic safety of herbal wound-healing formulations. Therefore, this study aimed to evaluate the 28-day repeated-dose oral toxicity of Khaolaor Ya-Kwad-Samarn-Lin (KLO), a Thai herbal formulation. The experimental protocol was adapted from OECD Test Guideline 407, and KLO was administered once daily by oral gavage at doses of 0.1, 1, and 5 mg/kg body weight/day. Mortality, clinical signs, behavioral changes, body-weight gain, food and water consumption, relative organ weights, gross necropsy findings, and histopathological changes were assessed. No mortality, treatment-related clinical signs, behavioral abnormalities, changes in body-weight gain, food or water consumption, or relative organ weights were observed in KLO-treated rats. Gross necropsy and histopathology revealed no treatment-related lesions. Isolated hematological changes were observed in high-dose male rats, including decreased mean corpuscular volume and granulocyte percentage; however, these changes showed no clear dose-response relationship and were not supported by clinical observations, organ-weight changes, gross pathological findings, or histopathological evidence. Overall, repeated oral administration of KLO did not produce detectable treatment-related adverse systemic toxicity. The no-observed-adverse-effect level (NOAEL) for KLO was determined to be 5 mg/kg body weight/day, the highest dose tested.

Keywords: Khaolaor Ya-Kwad-Samarn-Lin, Ya-Samarn-Phlae, Subacute toxicity, NOAEL

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Introduction

Herbal formulation intended for wound management have attracted increasing scientific interest because many plant-derived preparations exhibit multiple biological activities relevant to tissue repair, including antimicrobial, anti-inflammatory, antioxidant, and pro-healing effects [1, 2]. However, evidence of pharmacological efficacy alone is insufficient to support further development, particularly when herbal preparation may be administered repeatedly or used over extended periods. From a toxicological perspective, repeated-dose systemic safety assessment is necessary to identify potential target-organ toxicity, characterize treatment-related adverse effects, and support determination of the no-observed-adverse-effect level (NOAEL) [3, 4]. A 28-day repeated-dose oral toxicity study is therefore an important preclinical approach for evaluating the systemic toxicological profile of test articles intended for continued development[4].

Khaolaor Ya-Kwad-Samarn-Lin (KLO) is a traditional Thai herbal formulation used in the context of aphthous ulceration and inflammatory lesions of the oral cavity. According to the original instructions, KLO is mixed with a small amount of cooled boiled water and applied to the tongue two to three times daily. Although the exact dose and treatment duration are not specified, repeated application and potential incidental ingestion highlight the importance of evaluating its safety following repeated exposure. Earlier work on KLO-related aphthous ulcer remedies described a formulation containing *Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis*, and *Glycyrrhiza uralensis*, and reported low acute toxicity in animal models[5]. Traditional Thai remedies based on this formulation have also been reported to possess antimicrobial and antioxidant activities relevant to oral ulcer management[6]. More recently, KLO was incorporated into a durian gum-alginate mucoadhesive bilayer patch for oral ulcer treatment, and the optimized formulation showed favorable stability together with in vivo safety and wound-healing applicability in an oral ulcer model[7]. There is currently no research data available on repeated-dose systemic safety. A review of the literature reveals a lack of data regarding the subacute oral toxicity of the KLO formulation in experimental animals. Therefore, the present study aims to investigate this information. However, currently available research has focused predominantly on efficacy-related features, formulation development, and, in some cases, acute safety, whereas comparative subacute systemic safety profiles following

repeated oral administration remain insufficiently characterized. Therefore, the present study aimed to evaluate and compare the 28-day repeated-dose oral toxicity profiles of KLO extract in male and female Wistar rats using a study design adapted from OECD Test Guideline 407 [4]. The study assessed clinical observations, growth-related parameters, organ weights, serum biochemistry, hematology, gross pathology, and histopathology to determine whether repeated administration produced treatment-related adverse systemic effects and to establish NOAEL values for KLO extract.

Materials and Methods

Animals and ethical approval

A 28-day oral toxicity study with repeated doses was performed at the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand, utilizing a procedure modified from OECD Guideline 407. The study protocol received approval from the Institutional Animal Care and Use Committee (IACUC) under Approval No. MHESI 68014/1436; Ref. AR 065/2022, dated August 23, 2022.

Healthy Wistar rats of both sexes were used in this study. Male rats weighed 250-300 g, whereas female rats weighed 200-250 g at the beginning of the experiment. The animals were housed under controlled environmental conditions at a temperature of 22 ± 2 °C, relative humidity of 55 ± 10%, and a 12 h light/dark cycle. Animals were provided with standard pellet diet and reverse osmosis (RO)-filtered water ad libitum. The animal room was maintained under a strict hygienic conventional (SHC) system with adequate ventilation, minimal noise disturbance, and no unpleasant odors. Sterilized corn cob bedding was used as cage bedding material.

Prior to the experiment, all animals were acclimatized for 1 week to adapt to the laboratory environment. Prior to treatment initiation, all animals were acclimatized to handling and oral gavage procedures by receiving distilled water once daily for four consecutive days in order to minimize procedure-related stress.

Experimental design and treatment

The KLO formulation, containing *Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis*, and *Glycyrrhiza uralensis* and other, was extracted using microwave-assisted extraction (MAE) by suspending 15 g of the formulation in 60 mL of 95% ethanol. The chemical constituents of the extract were analyzed using liquid chromatography-mass spectrometry (LC-

MS), and the major marker compounds of the KLO formulation, namely gallic acid and berberine, were quantified using high-performance liquid chromatography (HPLC)[7].

The KLO extract was tested in the animals that randomly allocated into four groups, with eight rats per group, comprising four males and four females. The control group was given distilled water and the treatment group was administered the respective extract at 0.1, 1 and 5 mg/kg body weight/day. After the acclimatization period all the animals were randomly assigned to experimental groups. Animals in each experimental group were housed together in the same cage to minimize stress and to facilitate adaptation before the start of treatment. Individual animals were identified by noninvasive tail markings with permanent marker pens for monitoring and record keeping. All treatments were given once daily by oral gavage for 28 consecutive days. The study design was adapted from OECD Guideline 407 to evaluate the subacute oral toxicity profile of extract under repeated-dose exposure conditions.

Clinical observations and gross examination

All animals were observed daily during the treatment period for mortality, clinical signs of toxicity, behavioral abnormalities, and changes in physical appearance. Body weight, food consumption, and water intake were measured throughout the study to evaluate general health status and growth. Body weights were recorded daily at the same time throughout the experimental period until euthanasia.

At the end of the experimental period, all animals were euthanized by intraperitoneal administration of an overdose of sterile thiopental sodium. A complete gross necropsy was subsequently performed to identify any visible abnormalities in the internal organs. Macroscopic examination included the heart, lungs, liver, spleen, kidneys, stomach, small intestine, large intestine, and reproductive organs.

Blood collection, organ weight determination, and histopathological evaluation

For biochemical and hematological analyses, animals were fasted overnight (12 h) prior to blood collection on day 29 following the 28-day repeated-dose treatment period. For biochemical and hematological tests, about 3 mL of blood was taken from each animal. The lungs, heart, liver, spleen, kidneys, and reproductive organs were cut out, carefully dried, and weighed to find out how much they weighed in relation to each other.

Relative organ weight was calculated according to the following equation:

Relative organ weight (g/1000 g BW) = [organ weight (g) × 1000] / body weight (g)

The relative organ weight was expressed per 1000 g body weight to normalize differences in body weight among experimental animals. After the organs were collected, the tissues were preserved in 10% formalin for later histological examination.

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (mean ± SD) and analyzed separately according to extract type and sex. Comparisons between the control group and the extract-treated groups at different dose levels within each sex of each experiment were performed using one-way analysis of variance (one-way ANOVA), followed by Tukey's post hoc test when an overall difference was detected. Statistical significance was set at $p < 0.05$. Mortality, clinical signs, gross necropsy findings, and histopathological observations were presented descriptively.

Result

Clinical observations, survival, body weight, food and water intake, and relative organ weights

Daily oral administration of KLO extract for 28 consecutive days did not result in mortality in any treatment group, and all animals survived until the scheduled termination. No abnormal clinical signs, behavioral changes, or alterations in physical appearance were observed throughout the experimental period. Body weight gain, food consumption, and water intake in KLO-treated rats were comparable to those of the vehicle control group, with no statistically significant differences detected during the study period ($p > 0.05$). Relative organ weights also showed no statistically significant differences between the KLO-treated and control groups ($p > 0.05$).

Serum biochemical parameters

Serum biochemical parameters related to renal and hepatic function were evaluated in both sexes after 28 days of KLO administration. In male and female rats, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were comparable to those of the control group at all tested doses, with no statistically

significant differences observed ($p > 0.05$) (Table 1).

Hematological parameters

In male rats, no statistically significant differences were observed in red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), total white blood cell count (WBC), or monocyte percentage relative to the control group ($p > 0.05$). However, the high-dose group (5 mg/kg BW/day) showed a significantly lower mean corpuscular volume (MCV) and granulocyte percentage, than the control group ($p < 0.05$). In female rats, none of the evaluated hematological parameters, including RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, WBC, lymphocyte percentage, monocyte percentage, and granulocyte percentage, differed significantly from the vehicle control at any tested dose ($p > 0.05$) (Table 2).

Gross necropsy and histopathological findings

Gross necropsy performed at the end of the study revealed no macroscopic abnormalities in the examined internal organs, including the heart, lungs, liver, spleen, kidneys, stomach, small intestine, large intestine, and reproductive organs. All examined organs appeared normal in size, color, and consistency in all groups. Histopathological examination of all evaluated organs showed no abnormal microscopic findings in the heart, lungs, liver, spleen, kidneys, stomach, small intestine, large intestine, or reproductive organs of KLO-treated rats compared with the control group. The overall tissue architecture of all examined organs was preserved. In the liver, hepatocytes were arranged in hepatic cords surrounding the central vein. In the kidneys, glomeruli and Bowman’s capsules were intact, and no glomerular or tubular degeneration was observed. In addition, no edema, necrosis, congestion, hemorrhage, or inflammatory cell infiltration was observed in any of the examined tissues (Figures 1 and 2).

Table 1. Serum biochemical parameters of male and female rats following 28-day oral administration of KLO

Parameters	Male				Female			
	Control	**Low**	**Medium**	**High**	**Control**	**Low**	**Medium**	**High**
BUN	29.91±2.84	29.10±1.42	29.90±1.70	28.28±3.39	21.41±0.85	22.02±2.32	21.91±2.04	22.41±1.84
Creatinine	0.52 ± 0.03	0.53 ± 0.01	0.52 ± 0.04	0.52±0.05	0.57±0.02	0.59±0.05	0.57±0.00	0.55±0.02
AST	100.00±5.94	106.25±12.42	106.50±4.12	102.75±3.59	103.00±3.76	103.75±11.87	107.25±7.93	100.50±6.35
ALP	182.75±23.01	193.00±10.39	175.75±25.77	165.25±18.28	118.50±10.72	113.25±20.68	107.00±29.63	108.00±26.87

Values are expressed as mean ± SD (n = 4). The control group received water. KLO low-, medium-, and high-dose groups received 0.1, 1, and 5 mg/kg BW/day, respectively. Asterisks indicate statistically significant differences compared with the control group ($^{*}p < 0.05$; one-way ANOVA followed by Tukey's post hoc test)

Table 2. Hematological parameters of male and female rats following 28-day oral administration of KLO

Parameters	Male				Female			
	Control	Low	Medium	High	Control	Low	Medium	High
RBC	6.39±1.80	7.56±2.11	7.35±1.59	5.00±0.55	5.03±0.64	5.16±0.45	5.12±0.49	5.03±0.68
HGB	14.03±3.79	14.30±4.25	14.15±3.32	13.98±10.07	9.35±0.82	9.88±0.99	9.80±1.01	9.70±1.05
HCT	36.13±10.19	35.20±8.83	35.45±9.77	34.33±14.81	28.05±2.86	27.33±2.32	28.60±3.21	29.68±1.99
MCV	56.63±0.46	56.00±1.06	56.85±1.08	54.68±1.17*	57.10±0.99	56.40±0.88	57.05±1.52	57.03±1.39
MCH	18.53±0.46	18.55±0.47	18.73±0.51	17.95±0.24	19.33±0.81	19.05±0.35	19.53±0.72	19.30±0.84
MCHC	32.80 ±0.66	33.25±0.95	33.50±0.39	32.95±0.44	33.33±0.61	34.05±0.42	34.23±0.39	33.98±0.63
RDW	11.08±0.86	11.55±0.87	11.23±0.46	11.00±0.52	10.43±0.33	10.30±0.35	10.58±0.29	10.55±0.19
PLT	578.00±155.62	556.25±193.14	542.25±161.97	393.50±41.88	507.75±114.91	534.50±121.31	381.50±25.57	448.00±112.75
MPV	6.05±0.24	6.05±0.17	6.45±0.24	6.43±0.21	6.83±0.24	6.33±0.26	6.43±0.32	6.45±0.10
WBC	2.53±0.64	2.40±1.49	2.68±1.04	2.85±0.70	2.08±0.60	1.90±0.76	1.18±0.51	1.75±0.21
Lymph	77.75±3.04	79.20±2.72	80.48±1.00	82.93±3.23	82.68±3.34	80.20±1.81	80.60±0.97	81.18±2.13
Mon	1.93±0.29	2.03±0.30	1.95±0.24	2.08±0.88	2.18±0.69	2.15±0.29	1.93±0.17	2.13±0.30
Gran	20.05±2.53	16.88±1.78	17.45±0.84	14.63±2.73*	15.15±2.69	17.70±1.54	17.38±0.76	17.88±1.04

Values are expressed as mean ± SD (n = 4). The control group received water. KLO low-, medium-, and high-dose groups received 0.1, 1, and 5 mg/kg BW/day, respectively. Asterisks indicate statistically significant differences compared with the control group (*$p < 0.05$; one-way ANOVA followed by Tukey's post hoc test)

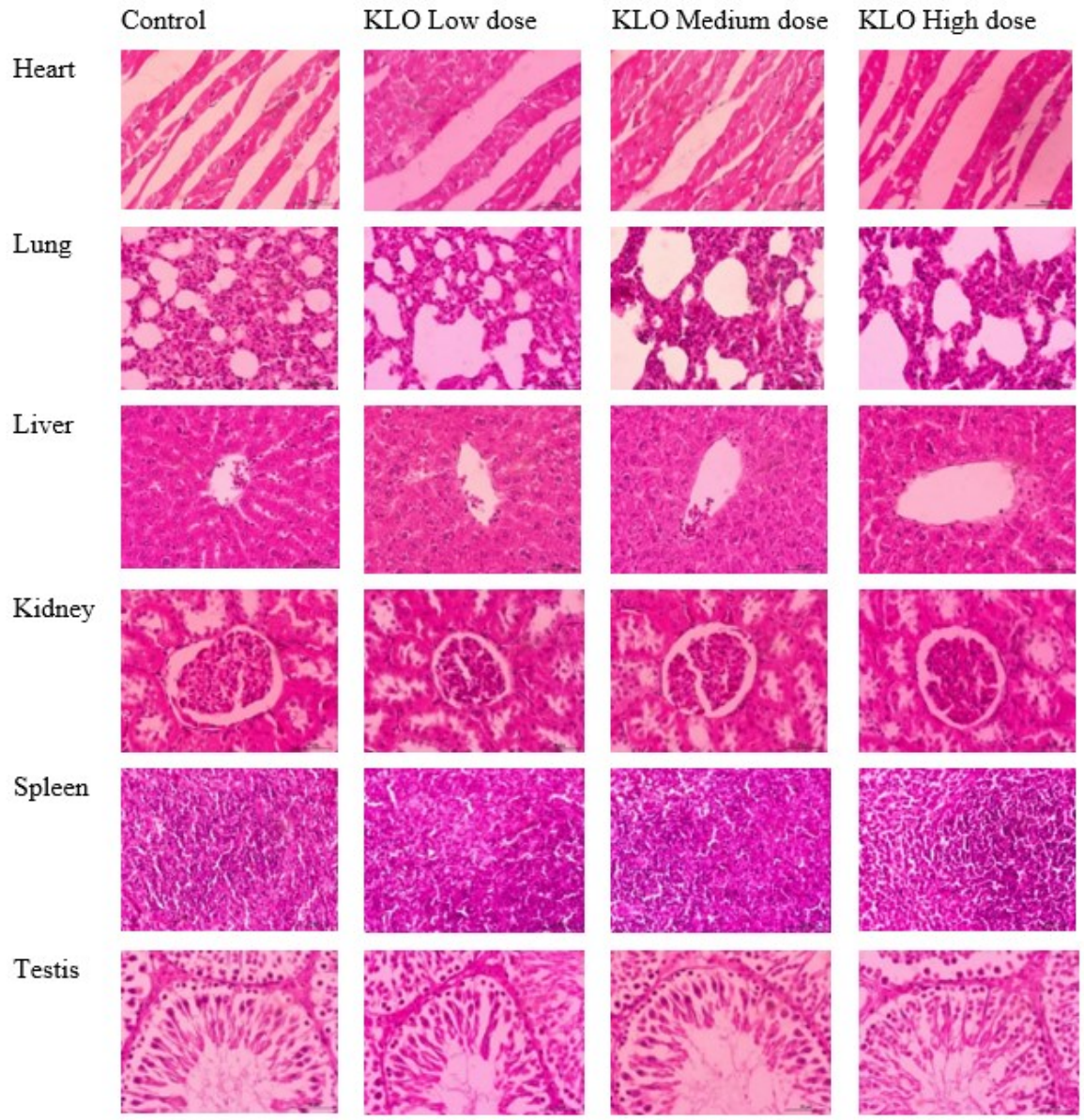


Figure 1. Representative histopathological images of the heart, lung, liver, kidney, spleen, and testis from male Wistar rats following 28-day oral administration of KLO (40× magnification; scale bar = 50 µm) (n=4) (KLO low-, medium-, and high-dose groups received 0.1, 1, and 5 mg/kg BW/day, respectively).

Discussion

The present study evaluated the systemic toxicological profile of KLO following repeated oral administration for 28 consecutive days in male and female Wistar rats. Daily administration of KLO did not result in mortality, treatment-related clinical signs, behavioral changes, altered body-weight gain, changes in food or water consumption, or significant changes in relative organ weights. Serum biochemical markers associated with hepatic and renal

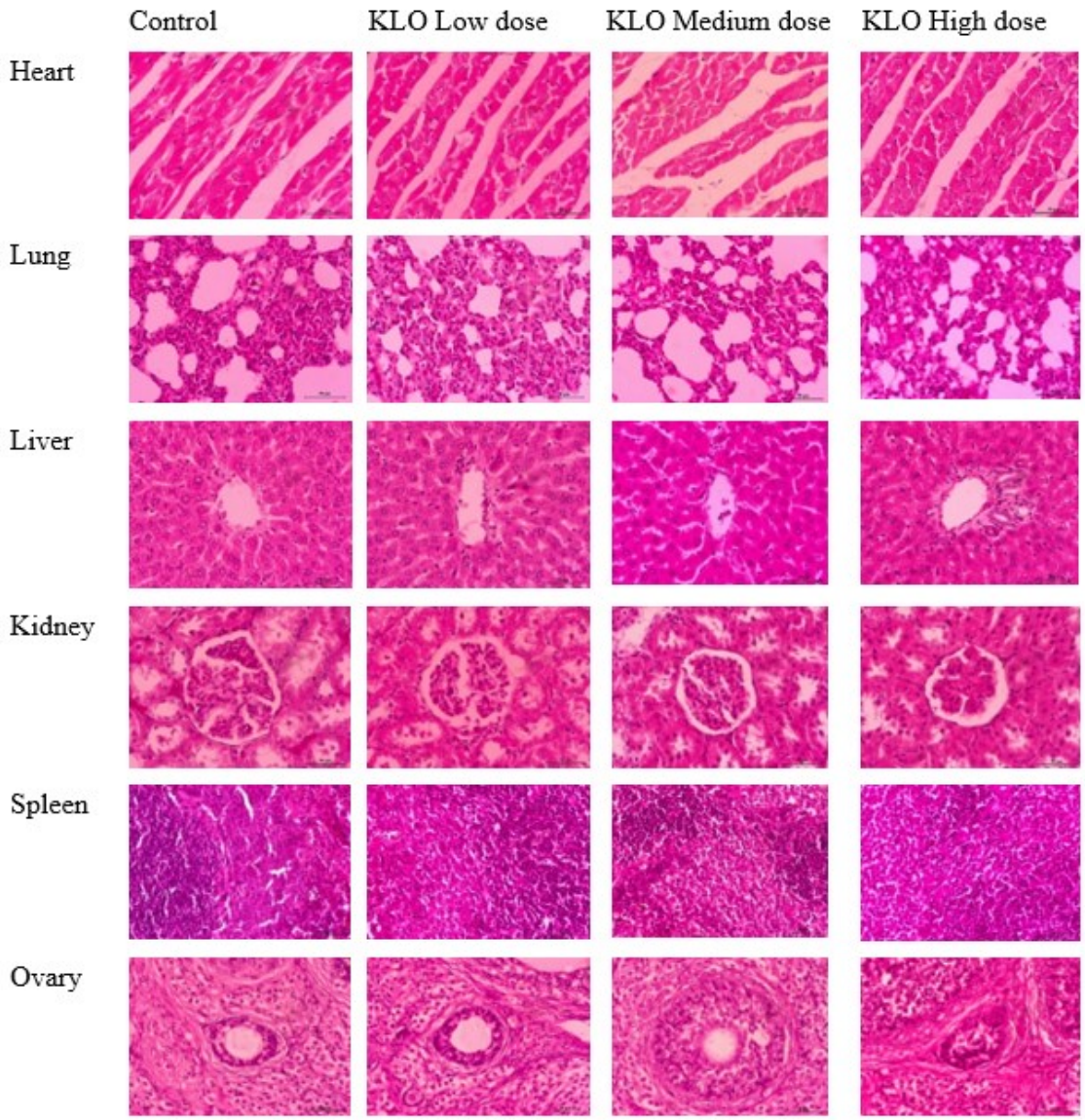


Figure 2. Representative histopathological images of the heart, lung, liver, kidney, spleen, and ovary from female Wistar rats following 28-day oral administration of KLO (40× magnification; scale bar = 50 µm) (n=4) (KLO low-, medium-, and high-dose groups received 0.1, 1, and 5 mg/kg BW/day, respectively).

function, including BUN, creatinine, AST, and ALP were not significantly altered in either sex. In addition, gross necropsy and histopathological examination revealed no treatment-related macroscopic or microscopic lesions in the examined organs. These findings are consistent with the purpose of a 28-day repeated-dose oral toxicity study, which is designed to identify adverse effects associated with repeated exposure and to support determination of the NOAEL of the test substance[4].

The main statistically significant findings in the KLO study were limited to high-dose male rats and consisted of decreased mean corpuscular volume, and granulocyte percentage. These findings were not accompanied by changes in total white blood cell count, erythrocyte count, hemoglobin, hematocrit, platelet count, serum biochemistry, relative organ weights, gross pathology, or histopathology. In nonclinical toxicology, the interpretation of clinical pathology findings should be based on concordance across multiple endpoints rather than statistical significance alone, because isolated clinical pathology changes may not necessarily indicate an adverse toxicological effect when they are not supported by related anatomical pathology or other toxicological endpoints[8,9]. Accordingly, the limited hematological changes observed in high-dose male rats were considered insufficient to establish a treatment-related adverse effect under the conditions of the present study.

The absence of hepatic and renal biochemical alterations, together with preserved tissue architecture in the liver, kidneys, and other examined organs, indicates that repeated oral administration of KLO did not produce detectable target-organ toxicity under the present experimental conditions. These findings are consistent with previously reported safety data for KLO-related formulation and selected constituent herbs. Watthanachai-yingcharoen *et al*. (2009) reported low acute toxicity of medicinal plants and herbal remedies used for aphthous ulcer management[5]. Additional support is provided by toxicological studies of *Kaempferia galanga*, an important constituent of KLO-related formulation. Repeated oral administration of ethanolic rhizome extract of *K. galanga* for 28 days produced no mortality, no significant changes in body or organ weights, and no gross or histopathological abnormalities[10]. Another study also reported that *K. galanga* rhizome extract was well tolerated after both acute and repeated oral exposure, with no overt toxicity observed at the tested dose levels[11]. Collectively, these observations support the perspective that essential elements of the KLO formulation exhibit safety profiles aligned with the subacute toxicity results identified in the current investigation.

Based on the integrated assessment of survival, clinical observations, growth-related parameters, serum biochemistry, hematology, relative organ weights, gross necropsy, and histopathology, KLO did not demonstrate treatment-related adverse systemic toxicity in this 28-day study. Although selected hematological changes were observed in high-dose male rats, these

changes lacked supporting evidence from other toxicological endpoints and were therefore not considered adverse[4,8,9]. Although the KLO is intended for topical application to oral ulcers, its use as a tongue-painting or oral swabbing preparation may result in some of the formulation dissolving and being swallowed into the gastrointestinal tract. Therefore, the 28-day repeated-dose oral toxicity study provides preliminary information on the systemic safety of the KLO formulation under the experimental conditions investigated. The NOAEL obtained in this study was 5 mg/kg body weight/day, representing the highest dose tested at which no adverse effects were observed under the experimental conditions. The limitations of extrapolating these findings to the safety of the product under its intended conditions of use, which typically involve a treatment period of approximately 7-14 days, have also been discussed. However, the repeated-dose oral toxicity study does not directly establish the local safety of the product following application to the oral mucosa. Therefore, further studies that better reflect the intended use of the product are warranted, including assessments of local oral mucosal toxicity and safety following topical application, to provide a more comprehensive safety profile[4].

Conclusion

In this 28-day repeated-dose oral toxicity study, KLO was well tolerated in male and female Wistar rats under the conditions tested. The KLO extract did not cause mortality, treatment-related clinical signs, altered growth-related parameters, significant changes in relative organ weights, or gross or microscopic pathological lesions. Isolated biochemical and hematological changes were observed in some treatment groups; however, these findings were not dose-dependent and were not supported by corresponding changes in organ weights, gross necropsy, or histopathology. Therefore, they were considered insufficient to indicate treatment-related adverse systemic toxicity.

The NOAEL values were observed to be 5 mg/kg body weight/day for KLO, which corresponded to the highest tested dose. The NOAEL observed within the dose range tested and under the specific experimental conditions of this study. These results provide preclinical repeated-dose systemic safety evidence for further toxicological and product-development evaluation of herbal wound-healing formulation.

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Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the results or interpretation of the work presented in this study.

Declaration of Generative AI and AI-assisted Technologies in the Manuscript preparation process

During the preparation of this work the authors used ChatGPT in order to assist with language refinement and grammatical editing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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