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Evaluation of the acute and sub-acute toxicity of the standardized extract of *Avicennia officinalis* L. in mice

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Abstract. *Avicennia officinalis* L. (AOL) has shown promise for its anti-inflammatory, anti-cancer, and antioxidant properties. However, there is no research on the toxicity of this plant in Vietnam. The acute and sub-acute toxicology study proved that AOL leaf extracts are practically non-toxic in normal mice. Acute toxicity assessment was conducted with single oral doses of AOL extract (2500, 3100, 4100, and 5000 mg/kg). In sub-acute toxicity, mice were administered daily oral doses of AOL extract (200 and 400 mg/kg) for 28 days. Blood was collected from the heart, liver, and kidneys for further analysis. The comprehensive results revealed an oral median lethal dose (LD_{50}) exceeding 5000 mg/kg, indicating low acute toxicity. A sub-acute study (200 and 400 mg/kg/day) showed no deaths or weight gain. At both doses, the standardized AOL extracts decreased serum aspartate transaminase activity. Administration of the 200 mg/kg group significantly increased blood urea levels; however, histological examination of mouse kidneys in this group revealed no signal of damage. Histological examination of mouse livers revealed mild degeneration, possibly due to the use of adult mice and potentially unrelated to the extract. This study emphasizes AOL's potential for further pharmacological testing based on its low acute toxicity and promising results.

Keywords: *Biochemical analysis, Hematological analysis, Swiss albino, and Traditional medicines*

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1. Introduction

Traditional medicines, the most ancient kind of healthcare, are employed for the prevention and treatment of both physical and mental ailments. Throughout history, several communities have devised pragmatic therapeutic techniques to tackle a range of health conditions and sometimes fatal diseases. Traditional medicines, usually referred to as complementary and alternative medicine or ethnic medicine, continue to have a significant impact in numerous nations (Yuan et al., 2016).

Unlike synthetic drugs, which undergo rigorous safety testing, the effectiveness and safety of herbal remedies haven't been definitively established through scientific research. Therefore, acute and sub-acute toxicity tests are routinely performed to investigate natural products or drugs (Bhardwaj & Gupta, 2012).

According to the European Pharmacopoeia, standardized extracts are extracts where the content of one or more known active ingredients has been deliberately adjusted to a specific concentration (European Directorate for the Quality of Medicines and Healthcare, 2013). Moreover, AOL leaves have a rich history of use in traditional medicine. The leaves of this plant have a lot of steroids, tannins, saponins, and phenols (Ali, 2006; Thatoi et al., 2016). They may help with ulcers, pain, inflammation, cancer, and free radicals (Bandaranayake, 2002; Mollah & Das, 2024).

This study focuses on the well-characterized and standardized Avicennia officinalis L. (AOL) extract. This extract serves as the foundation for developing high-quality medicinal herb-derived drugs. Furthermore, there has been no research on the toxicity of this plant in Vietnam (Figure 1). Despite the lack of scientific safety evaluation, AOL has potential medicinal uses. To establish a safe dosage for further research, this study investigated the acute and sub-acute toxicity of AOL standardized extract in Swiss

Albino mice. This information is crucial for determining safe doses in future studies exploring the herb's pharmacological properties and biological effects.

Figure 1. *Avicenia officinalis* trees and leaves in Ngoc Hien district, Ca Mau province

2. Materials and Methods

2.1. Material

Leaves of AOL were gathered from the Ngoc Hien area, Ca Mau province, in July 2023. The leaves were rinsed with water to eliminate soil and dirt. The moisture content of the dried sample was less than 13%. Samples were housed in black glass containers with silica gel and maintained at ambient temperature. The plants were discovered using polymerase chain reaction (PCR) techniques at the Department of Biology, Can Tho University, Vietnam.

2.2. Preparation of plant extract

The dried sample had moisture content below 13%. 100 g of AOL leaves were extracted with 1900 mL ethanol 50°C. The extract was heat refluxed at temperature of 66°C for 2 hours. The resulting extract was evaporated under vacuum, at a temperature not exceeding 55°C, until almost free from solvent. The AOL standardized extract quantified the total phenolic content of 19 µg/mL and total flavonoid content of 50 µg/mL.

2.3. Experimental animals

Swiss Albino mice of both sexes (25±2 g, 6–8 weeks old) were obtained from the Pasteur Institute in Ho Chi Minh City. The experiment was carried out at Can Tho University of Medicine and Pharmacy. Mice were acclimated to the laboratory environment for 7 days before the experiment. During this time, the mice were housed in groups of ten in cages measuring 41 × 27 × 14.5 cm. Food and water were constantly available.

2.4. Acute oral toxicity

Acute oral toxicity of the extract was studied in mice following the “Guidelines for preclinical and clinical trials of oriental and herbal medicines” of the Ministry of Health Vietnam (Ministry of Health Vietnam, 2015) and Guideline No. 425 of the Organization for Economic Cooperation and Development (OECD, 2001). The 'Up-and-Down' test method was used with a single dose administered to the mice.

Before the experiment, the mice were fasted for 16 hours with access to water. The mice received the test substance at a dose of 0.1 mL/10 g of body weight three times in 24 hours. Each dose was administered three hours apart. The mice were monitored for general health (activity, intake, and excretion) throughout the 72 hours following the last administration of the test substance. If both mice survive, the dose might be increased. Conversely, if both die, the dose will be lowered. This process continues until a dose is found, during which one mouse survives and the other dies. The LD_{50} value (dose killing 50% of the experimental animals) is calculated using the Litchfield-Wilcoxon method (Ministry of Health Vietnam, 2015).

2.5. Sub-acute toxicity (28 days)

Mice of both sexes were assigned randomly to four groups (n = 10/group: five males and five females). Daily dosing by the proposed clinical route was performed at two dose levels: a low dose and a medium dose (double the low dose) (OECD, 2008). These two dose levels were selected for use in studying the pharmacological effects of AOL extract (Sumithra et al., 2011; Islam et al., 2022). Group I (control) received distilled water only. Group II (Tween 80) received 5% Tween 80 solvent. Groups III and IV received 200 and 400 mg/kg of the extract, respectively. The mice in all groups were dosed by oral gavage for 28 days. The mice were weighed weekly and observed for toxic reactions and mortality.

After receiving the treatment for 28 days, the mice were weighed and euthanized. Blood samples were collected via cardiac puncture for precise hematological (red blood cell, hemoglobin, hematocrit, platelet, white blood cells, mean platelet volume, procalcitonin) and biochemical analyses of the liver (AST, ALT) and kidneys (creatinine, blood urea). The blood samples were then analyzed at the Medlatec Clinic in Can Tho City, a facility known for its accuracy and reliability in medical testing. Following excision, the liver and kidneys were weighed, photographed, and fixed in 10% formalin for meticulous histological analysis with hematoxylin-eosin staining, ensuring a thorough examination of any potential effects of the AOL extract. A hematoxylin and eosin solution was applied to stain the tissue for a permanent slide that can be analyzed under a microscope.

2.6. Statistical analyses

Statistical analysis was conducted using IBM SPSS 27 software and Microsoft Office Excel. The data values were reported as the mean ± standard error of the mean (SEM). In the case of regularly distributed data, a one-way ANOVA analysis was conducted, followed by Dunnett's comparison test to compare each group with the control group. The Mann-Whitney U test was employed to compare two groups with non- normally distributed data. Results were deemed statistically significant when the p-value was less than 0.05.

3. Results and Discussion

3.1. Acute toxicity

3.1.1. Dose-finding phase

This study proposes starting with a single oral dose of AOL at 2500 mg/kg body weight in two healthy mice. After 72 hours, both mice were alive and showed no signs of harmful effects. Continue testing the following single doses: 3100 mg/kg, 4100 mg/kg, and 5000 mg/kg body weight. The 5000 mg/kg dose is the maximum dose that can be given to mice without dying, so stop exploring the dose.

3.1.2. Determination phase

A total of 24 mice were randomly allocated into 3 groups of 8 mice per group. The groups were Group I (control), Group II (5% Tween 80 solution), and Group III (AOL 5000 mg/kg). Observation for 72 hours after giving the mice water revealed no deaths in any research group. After 3 days, an autopsy of the mice showed normal liver and kidney appearance (smooth surface, dark red, no congestion or changes) (Figure 2). The oral LD50 of AOL extract in mice was more significant than 5000 mg/kg for both males and females.

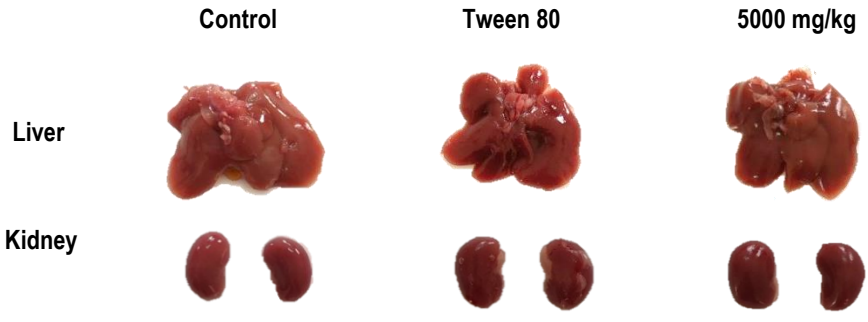

Figure 2. Macroscopic livers and kidneys of mice in the acute toxicity study of AOL extract

Drugs intended for humans typically undergo pre-clinical testing in animals to assess their effects following both single (acute) and repeated dosing. This helps understand the potential impact of the drug on a living organism after both short- and long-term exposure. In this study, the extract was administered to the mice orally to evaluate its potential toxicity since it is the most common route for drug delivery.

In Vietnam, there has been no research on the acute toxicity of this AOL plant. Research data on the toxicity of AOL extract in the world is still limited; most of it comes from Bangladesh and India. For instance, in Bangladesh, two separate acute toxicity studies investigated the effects of the extract on mice at doses of 3000 mg/kg (Sumithra et al., 2011) and 3200 mg/kg (Aunjum et al., 2021), respectively. Neither study observed any signs of toxicity or death in the animals. To gain more information on the safety profile, this study investigated the acute toxicity of AOL extract at higher doses than those used in previous studies. This study followed OECD guideline 425 for acute oral toxicity testing, adhering to the Ministry of Health Vietnam's instructions. The starting dose was 2500 mg/kg of body weight, half the anticipated initial dose. Subsequent dose adjustments were made using the "up-and-down" method described in OECD Guideline 425.

The results indicated that the LD_{50} of the AOL extract did not reach the tested range. Since no mortality was observed at the highest dose of 5000 mg/kg, the extract can be tentatively classified under Category 5 (low toxicity) according to the Globally Harmonized Classification System for Chemical Substances and Mixtures. However, studies on acute toxicity often do not give enough information about long-term effects. So, this study examined effects over a more extended period (sub-acute) to better understand them.

3.2. Sub-acute toxicity (28 days)

3.2.1. Body weight of mice

Over the 4-week experiment, mice in all groups acted normally, ate well, had smooth fur, and had no dead mice or abnormal symptoms. All groups experienced a modest weight decrease in week 1, followed by a gradual weekly gain of approximately 2 grams. By the end of the study, there were no statistically significant differences ($p > 0.05$) in body weight between the treatment groups (200 mg/kg, 400 mg/kg, and 5% Tween 80) and the control group (Figure 3). No significant weight gain was observed during the study, and food and water consumption remained normal. Body weight changes from the study showed normal growth in all four groups of mice, with no statistically significant differences in weight gain between groups. Daily health monitoring also showed no abnormal clinical signs in any group of mice.

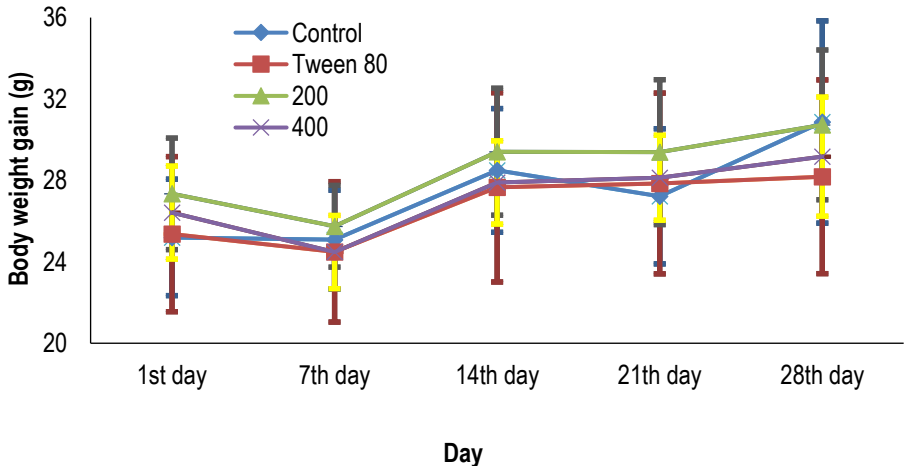

Figure 3. Changes in body weight of mice following administration of AOL extract for 28 days. Values are presented as Mean ± SEM (n = 10). One-way ANOVA, compared with control.

3.2.2. Hematological parameters

The hematopoietic system is a sensitive target for toxicants, reflecting both pathological and physiological changes. The hematological analysis results showed no statistically significant changes ($p > 0.05$) in other blood hematological examinations

compared to the control group. The data are shown in Table 1. Hematological analysis of the AOL extract group revealed no significant changes in parameters such as white blood cell count, hemoglobin, red blood cell count, MCV, platelet count, and hematocrit value compared to the control group. This suggested that AOL did not affect the blood cell profile of the treated animals.

Table 1. Effect of AOL on hematological parameters in mice.

Parameters	Control	Tween 80	200 mg/kg doses	400 mg/kg doses
RBC (Tera/L)	8.62 ± 0.60	9.32 ± 0.75	8.61 ± 0.74	8.49 ± 0.98
HB (g/dL)	10.67 ± 4.50	12.96 ± 1.20	12.38 ± 0.82	11.90 ± 1.21
HCT (%)	42.10 ± 1.71	43.28 ± 4.46	41.30 ± 2.33	40.18 ± 3.77
PLT (G/L)	817.86 ± 293.02	707.00 ± 191.25	643.43 ± 85.41	724.43 ± 442.03
WBC (G/L)	11.34 ± 2.00	14.80 ± 8.48	7.27 ± 4.41	9.58 ± 2.77
MPV (fL)	6.57 ± 1.78	7.48 ± 3.11	6.76 ± 1.27	7.27 ± 3.92
PCT (%)	0.47 ± 0.96	0.39 ± 0.12	0.43 ± 0.69	0.45 ± 0.26

Note. Values are presented as Mean ± SEM (n = 7). Mann-Whitney U test compared with control. RBC, red blood cell; HB, hemoglobin; HCT, hematocrit; PLT, platelet; WBC, white blood cells; MPV, mean platelet volume; PCT, procalcitonin.

3.2.3. Biochemical parameters

Biochemical analysis revealed that AOL extracts at 200 and 400 mg/kg doses significantly reduced AST enzyme activity, suggesting an effect on liver function. However, the 200 mg/kg dose also caused a statistically significant increase in blood urea compared to the control group. Further details regarding the data are presented in Table 2.

Table 2. Effect of AOL on liver and kidney function in mice.

Parameters	Control	Tween 80	200 mg/kg doses	400 mg/kg doses
AST	222.57±131.05	136.43±47.65	94.86±17.42*	107.86±31.18*
ALT	49.86±16.33	40.43±4.99	43.43± 12.22	45.00 ± 25.84
Urea	5.07± 1.16	4.56±1.25	7.74±1.11*	5.80 ± 2.66
Creatinine	28.27 ± 2.16	28.27 ± 3.15	28.76 ± 1.11	27.77 ± 1.84

Note. Values are presented as Mean ± SEM (n = 7); Significant about at *p < 0.05; Mann-Whitney U test compared with control. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

The liver and kidneys play crucial roles in fundamental metabolic and excretory processes. Serum liver function tests provide insight into the liver's health. Measuring the activity of' marker' or diagnostic enzymes in tissues is important for diagnosing diseases, investigating diseases, and assessing the safety or toxicity risk of plant extracts (Yakubu, 2005). Increases in liver enzyme activity in the blood are a sign of liver cell damage (hepatocellular toxicity), according to Brautbar and Williams in 2002. Conversely, Akanji et al. (2013) suggested that decreasing levels of these enzymes could signify their blocking (enzyme inhibition).

After checking liver biochemical parameters, AST enzyme activity in the high-dose AOL test groups decreased significantly compared to the control group. Further investigation may focus on the potential antioxidant properties of AOL's hepatoprotective activity. Moreover, there was an increase in blood urea compared to the control group. The 200 mg/kg AOL dose group also showed a significant increase in blood urea levels compared to controls, suggesting potential kidney effects. However, elevated blood urea levels can have other explanations, such as changes in urine concentration or a high-protein diet (Yang & Lise, 2005).

3.2.4. Macroscopic liver and kidney

Examination of the mice at the end of the study (28 days) showed no differences in the size, color, or firmness of their livers and kidneys (Figure 4). By analyzing organ weight relative to body weight (organ weight ratio), researchers can gain valuable insights into how the AOL extract affects different organ systems in mice. This approach is a sensitive measure of potential physiological changes induced by the extract, as alterations in organ size or weight can indicate a response to

treatment or underlying toxicity. To calculate organ coefficients, livers were harvested from the mice, and the coefficient for each animal was determined using the following formula: Organ coefficient (g/100 g) = (absolute organ weight (g)/body weight on sacrifice day) × 100 (Kifayatullah et al., 2015). After 28 days of treatment, organ weight analysis revealed no statistically significant differences in the organ-to-body weight ratio between the control and AOL extract-treated groups (Table 3).

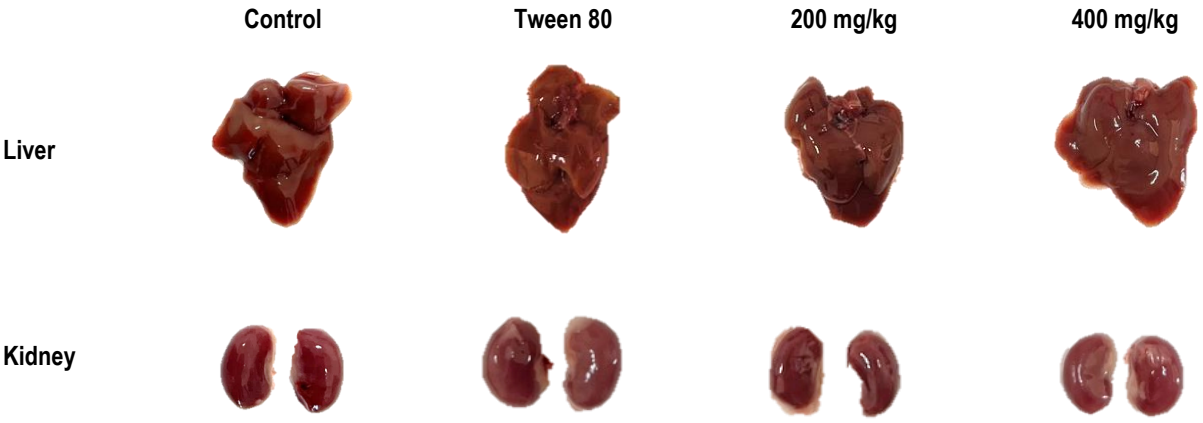

Figure 4. Macroscopic livers and kidneys of mice in the sub-acute toxicity study of AOL extract

Table 3. Effect of daily oral administration of AOL on liver in mice.

Organ \ Group	Control	Tween 80	200 mg/kg	400 mg/kg
Liver	4.2261 ± 0.32369	4.0079 ± 0.39964	4.1626 ± 0.71863	4.5710 ± 0.77032

Note. Values are presented as Mean ± SEM (n = 10); Mann-Whitney U test compared with control.

3.2.5. Histopathological study

Following the completion of treatment, a histopathological examination was conducted on important organs, such as the liver and kidney, to gather further data that supports the results obtained from the previous biochemical examinations. In all four groups of mice (Group I: Control, Group II: Tween 80 5%, Group III: 200 mg/kg AOL, and Group IV: 400 mg/kg AOL), histopathological studies showed mild degeneration (Figure 5). This degeneration had a minimal impact. Kidney structure in all groups was normal, with no signs of degeneration in renal tubular epithelial cells (Figure 6). These findings suggest that AOL extract has a minimal impact on mice's macroscopic and microscopic structures at the tested dose.

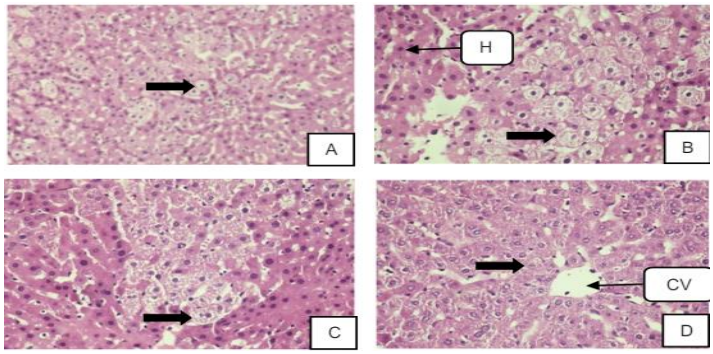

Figure 5. Effects of AOL extract on mice's liver tissue over a period of 28 days. (A) Liver tissues from control mice; (B) Liver tissues from mice treated with Tween 80 solvent; (C) Liver tissues from mice treated with 200 mg/kg AOL; and (D) Liver tissues from mice treated with 400 mg/kg AOL. Arrows indicate hepatocyte degeneration, which was observed in all groups. *Note*: CV = Central vein; H = Hepatocytes.

The liver is a very sensitive organ to toxic substances, drugs, or bioactive compounds because it metabolizes these substances into other compounds (Rhiouani et al., 2008). A histological examination of the kidneys revealed no abnormalities in any group. This finding suggests a lack of association between elevated blood urea and AOL extract administration. Histopathological studies of mouse livers from all groups revealed mild degeneration without inflammation or congestion. Histological examinations revealed that the AOL extract had a minimal impact on the mice's macroscopic and microscopic structures. Although mild liver cell degeneration was observed in all groups, this is likely due to the normal aging process in these mice. Adult mice (8 weeks old) were used, and after 4 weeks of the experiment, they would be entering their aging stage. Therefore, these changes are consistent with expected physiological development and are not necessarily an effect of the extract.

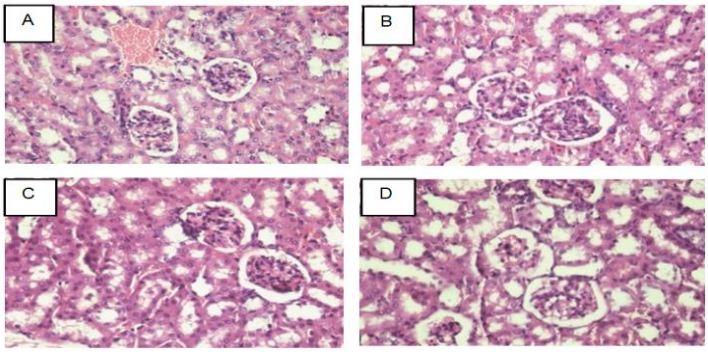

Figure 6. Effects of AOL extract on mice's kidney tissue over a period of 28 days. (A) Kidney tissues from control mice; (B) Kidney tissues from mice treated with Tween 80 solvent; (C) Kidney tissues from mice treated with 200 mg/kg AOL; and (D) Kidney tissues from mice treated with 400 mg/kg AOL. No signs of degeneration were observed.

4. Conclusions

The standardized AOL extract did not exhibit acute oral toxicity at a single 5000 mg/kg dose. However, specific effects on liver and kidney function were observed in sub-toxicity studies. According to this study, the standardized AOL extract has promise for further liver and kidney pharmacological testing. Additional investigations are warranted to assess its safety profile fully at different doses and with repeated exposure.

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