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Original Research Article

Effects of Pegagan Embun (*Hydrocotyle sibthorpioides* Lam) Extract on Renal Function in Male Wistar Rats as Assessed by Creatinine Clearance

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ABSTRACT

In Indonesia, pegagan embun (*Hydrocotyle sibthorpioides* Lam), a low-growing plant native to southeastern Asia, has been used as a traditional medicine to treat numerous ailments. However, additional research on the safety of this plant is needed, particularly before it can be promoted as a potential immune system-boosting drug. The present study aimed to evaluate the sub-acute toxicity effects of ethanol extract of *Hydrocotyle sibthorpioides* (HS) on renal function. Thirty-six male Wistar rats divided into four groups were used for this study. The control group was given NaCMC (0.5%), and the three treatment groups received an oral dose of the extract of HS at doses of 7, 35, and 150 mg/kg BW, respectively, for 21 days. The parameters measured include: 24-hour urine volume, serum creatinine, urine creatinine, creatinine clearance, and the percentage of renal function on the 7th, 14th, and 21st days were determined. The results showed that doses of HS extract at 7, 35, and 150 mg/kg BW significantly affected creatinine clearance (p<0.05). In contrast, the duration of administration has no significant effect on creatinine clearance (p>0.05). Renal function increased above the standard value (>100%) at 7 and 35 mg/kg BW doses. Thus, these concentrations could potentially cause impairments in renal function. The study concludes that various doses of the ethanol extract of *Hydrocotyle sibthorpioides* affected renal creatinine clearance in Wister rats. In contrast, the duration of administration of *Hydrocotyle sibthorpioides* extract did not affect creatinine clearance in the test animals.

Keywords: Creatinine, *Hydrocotyle sibthorpioides*, Pegagan Embun, Renal Function, Toxicity

Introduction

Indonesia possesses a rich biodiversity of germplasm, flora, and fauna that provides a potential source of natural medicine, many of which come from medicinal plants.[1] Traditional medicine has demonstrated health benefits in more than 80% of the global population, particularly in developing countries.[1]

One of these medicinal plants, pegagan embun (*Hydrocotyle sibthorpioides* Lam), a low-growing plant native to southeastern Asia, is commonly used in China as a traditional medicine.[2] *Hydrocotyle sibthorpioides* (HS) has been found to increase overall physical health and is used to treat liver-related problems and anaemia. In addition, extracts of HS have been shown to reduce swelling; exert anti-inflammatory, diuretic, antipyretic, and expectorant properties.[3,4,5] Moreover, a standardised extract of HS has been found to increase NK cell activity and CD8 cell numbers in male Wistar rats exposed to the H5N1 viral antigen.[6]

During the COVID-19 pandemic, *Hydrocotyle sibthorpioides* showed potential as a medicinal herb for increasing immune strength, most likely due to its immunostimulatory activity.[3] HS is known to contain flavonoid compounds that act as an antioxidant. Flavonoids are known to increase immune response and exert antagonist effects against lymphokines produced by T cells responsible for the stimulation of phagocytosis.[7]

According to regulations issued by the Ministry of Health in Indonesia, medicines developed for human use should be safe, show demonstrable health benefits, and be of high quality. To this end, the Ministry of Health Indonesia has issued various policies to ensure the safety and efficacy of traditional medicines.

The kidneys' primary function, a key target of toxic chemicals, is to eliminate metabolic waste products and excrete foreign compounds. Renal function is assessed by determining the creatinine clearance rate, a function of the amount of creatinine in the urine multiplied by the total volume of urine produced over 24 hours and then divided by the amount of creatinine in the blood. Creatinine is a waste product of muscle metabolism excreted in the urine, and low creatinine clearance indicates renal impairment. [8] Thus, high levels of creatinine are an indication of poor creatinine excretion and renal impairment.[9]

Toxicity testing is one of the pre-clinical requirements in developing traditional medicines. Hence, the present study aimed to determine the renal toxicity of *Hydrocotyle sibthorpioides* extract in Wistar rats.

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Material and Methods

Chemicals

Ethanol (70%), sodium carboxymethyl cellulose (NaCMC) (0.5%), aquadest, phytochemical screening reagents, rutin, Silica gel 60 F_{254} TLC Plate, animal food, creatinine analysis reagent (Greiner) consists of reagent I (sodium hydroxide 160 mmol/L) and reagent II (picric acid 4.0 mmol/L), and standard creatinine (2 mg/dL).

Animals

Healthy male Wistar rats (36) weighing 200 - 250 grams were used for this study. The animals were acclimatised for one week and provided

standard animal food and water *ad libitum.* Ethical approval for the test animals was obtained from the Faculty of Medicine Ethics Committee of Universitas Andalas, with approval number 403/UN.16.2/KEP-FK/2021.

Plant materials and collection
Fresh leaf samples of *Hydrocotyle sibthorpioides* were obtained from Jalan Bukit Ngalau, LubukKilangan sub-district, Indarung district, Padang City, West Sumatera. The plants were collected between May 1 and June 30, 2022. The plant sample was identified and validated by Dr Nurainas of Andalas University Herbarium (ANDA), Department of Biology, Faculty of Mathematics and Natural Science, Universitas Andalas, Padang City, West Sumatera. A voucher specimen number 247/K-ID/ANDA/V/2021 was assigned.

Preparation of the extract
Up to 5 kg of fresh plant sample was dried, and ground to a fine powder (650 g). This sample was subsequently macerated with 70% ethanol at a ratio of 1:10, sample-to-solvent, in a dark-coloured glass container for 6 hours. The filtrate was dried using a rotary evaporator at reduced pressure.[10]

Characterisation of the extract
The characterisation of the extract included nonspecific, specific, and chemical testing as follows: a) nonspecific testing measured the loss of weight on drying, total ash content, acid insoluble ash content, and water-soluble ash content; b) specific testing included organoleptic tests (i.e. shape, colour, taste, odour), parameter identify (i.e. names identified and compounds contained); and c) chemical testing determined the phytochemical components (e.g. alkaloids, saponins, phenols, flavonoids, steroids, and terpenoids), including thin-layer chromatography, and total flavonoid content determination.[10]

Sub-acute toxicity testing
The 36 male Wistar rats were weighed and divided into four groups: Group I (the control group) received NaCMC (0.5%). Groups II, III, and IV received oral doses of HS extract at 7, 35, and 150 mg/kg BW, respectively. The extract was administered orally once daily for 7, 14, and 21 days at 10.00 h. The volume of water administered to each animal daily was 50 mL. On the 8^{th}, 15^{th}, and 22^{nd} days, the animals were sacrificed, and their renal function parameters, including urine volume, urine creatinine, serum creatinine, creatinine clearance, and the percentage of renal function, were analysed.
24-hour urine volume measurement
Urine from the study animals was collected and measured with a measuring cylinder.

Measurement of serum creatinine
Serum creatinine was assayed on the 8^{th}, 15^{th}, and 22^{nd} day of extract administration and was obtained from the blood through the orbital eye sinus of the male Wistar rats. The blood sample was collected into a gel and clot activator tube and centrifuged at 3000rpm for 10 minutes. The serum was collected by micropipette and transferred into the microtube. The serum creatinine level was measured with a photometer 5010 v5+ by mixing 1 mL of each reagent I and II. A total of 50 µL serum was pipetted into the test tube, and 1mL of working reagent (mixtures of reagent I and II) was added. The serum creatinine was measured at a wavelength of 492 nm.[11]

Measurement of urine creatinine
The urine was diluted with aquadest (1:20) in the volumetric flask, and the creatinine levels were measured with a photometer 5010 v5+ by mixing 1 mL of each reagent I and II. A total of 50 µL serum was pipetted into the test tube, and 1 mL working reagent (mixtures of reagent I and II) was added. The urine creatinine was measured at a wavelength of 492 nm.[11]
Calculation of creatinine clearance
The creatinine clearance was calculated using the following equation.[12]

$$CrCl = \frac{Ucr\ x\ Uv}{Scr\ x\ t}$$

Abbreviations are defined as:
CrCl= Creatinine clearance (mL/min)
Ucr= Urine creatine (mg/dL)
Uv= 24-hour urine volume (mL)
Scr= Serum creatinine (mg/dL)
T= Time (1440 minutes)
Calculation of renal function (%)
The percentage of renal function was calculated using the following equation.[12]

$$\text{RF} = \frac{CrCl\ P}{Crcl\ K}\ x\ 100\ \%$$

Abbreviations are defined as:
RF= Renal function (%)
CrCl P = Creatinine clearance of extract-treated groups (mL/min)
CrCl K = Creatinine clearance of control group (mL/min)

Statistical analysis
The data were analysed by two-way ANOVA between the time (duration of administration) and doses. Subsequently, the significant results were analysed by Duncan's multiple range test (p<0.05) using IBM SPSS Statistics V24.

Results and Discussion

The ethanol extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) was analysed to determine its safety and quality. The nonspecific testing results are as follows: loss of weight on drying (9.18%), total ash content (8.82%), acid-insoluble ash content (0.05%), and water-soluble content (8.02%).
The organoleptic test showed that the extract was dark brown in colour and viscous, with a distinct smell and bitter taste. Phytochemical screening gave positive results for flavonoids, terpenoids, and saponins (Table 1).
Thin-layer chromatography (TLC) was carried out to identify the major chemical constituents of (*Hydrocotyle sibthorpioides* Lam).[13] The stationary phase was a Silica gel $60F_{254}$ TLC plate, and the mobile phase was a mixture of n-butanol, acetic acid, and water in a ratio of 4:1:5. The TLC plate was examined under UV light at a wavelength of 254 nm. The Rf value of the *Hydrocotyle sibthorpioides* ethanol extract and rutin was 0.60, indicating that the extract contains rutin (Figure 1).
Standardised analysis was used to determine the total flavonoid content of the HS extract, which was found to be 1.34%.

Urine volume
The results showed that the three different dose concentrations significantly affected urine volume over 24 hours (p<0.05). In contrast, the duration of administration (in days) had a non-significant effect (p>0.05). The results show that urine volume increased after the extract was administered (Figure 2). Flavonoids in the extract may have likely caused the increase in urine volume. Flavonoids have diuretic properties and may have been responsible for increased urine volume by increasing the glomerulus filtration rate (GFR). Flavonoids also inhibit the reabsorption of Na^+ and Cl^-, leading to increased Na^+ concentration and water in the tubules. The increased urine volume reflects in the tubules and the amount of urine excreted by the kidney.[14]

Table 1: Results of Phytochemical Screening

Phytochemical screening	Result
Alkaloid	-
Flavonoid	+
Phenolic	+
Saponin	+
Steroid	-
Terpenoid	+

(+) = contains a secondary metabolite
(–) = does not contain a secondary metabolite

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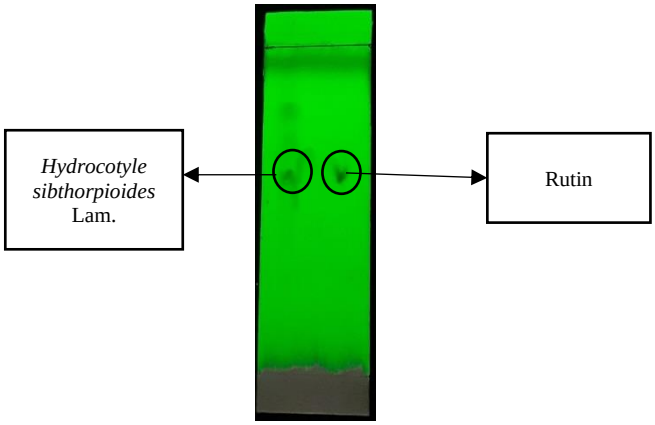

Figure 1: TLC profile of *Hydrocotyle sibthorpioides* Lam., viewed under 254 nm UV light

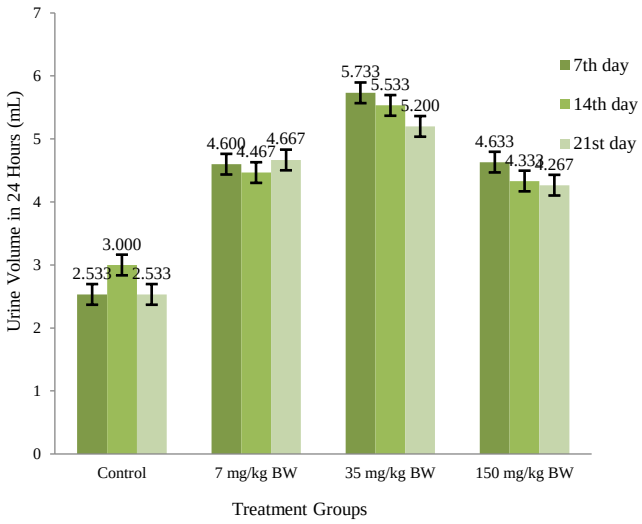

Figure 2: The effect of dose and duration of administration of *Hydrocotyle sibthorpioides* Lam extract on the 24-hour urine volume in male Wistar rats

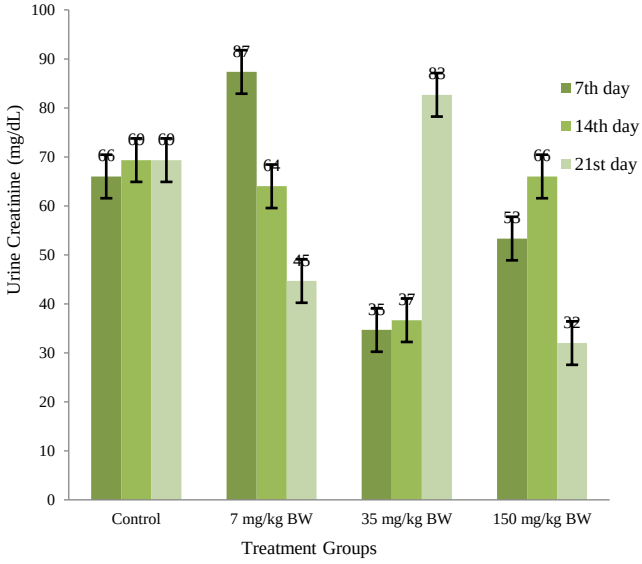

Figure 3: The effects of dose and duration of administration of *Hydrocotyle sibthorpioides* Lam extract on urine creatinine in male Wistar rats

Urine creatinine

As illustrated in Figure 3, concentrations of urine creatinine were significantly affected by the dose of HS extract administered ($p<0.05$), but were not significantly affected by the duration of administration ($p>0.05$). The results show a decrease in urine creatinine levels. The urine creatinine decreased proportionally with a decrease in the measured glomerular filtration rate (mGFR). A decrease in GFR was accompanied by a decrease in the number of nephrons, indicating renal impairment.

S

erum creatinine

As shown in Figure 4, serum creatinine was significantly affected by the dose and duration of administration ($p<0.05$). The observed increase in serum creatinine following oral doses of 7mg/kg and 35 mg/kg indicates renal impairment. Although Figure 4 shows possible renal impairment due to increased serum creatinine levels, the increase in serum creatinine overall was still in the normal range (0.578 - 1.128 mg/dL) for male Wistar rats.[8]

Creatinine clearance

Creatinine clearance was significantly affected by various doses ($p<0.05$), but it showed a non-significant effect on the duration of administration ($p>0.05$). A decrease in creatinine clearance is the main parameter that indicates a renal disorder. A higher serum creatinine level causes renal impairment more than the amount of creatinine excreted through the urine.[8] The volume of urine must be measured to determine creatinine clearance accurately. In the present study, urine volume increased when the extract was administered, thereby increasing creatinine clearance (Figure 5). The results showed that creatinine clearance was significantly affected by doses of 7 and 35 mg/kg BW but not by 150 mg/kg BW (i.e. the optimal dose for immunostimulatory activity).

Percentage of renal function

The percentage of renal function was significantly affected by the different doses of HS extract ($p<0.05$) in contrast to the duration of administration ($p>0.05$). The percentage of renal function increased above 100% in rats treated with HS extract compared to the control (Figure 6). This increase in percentage renal function resulted from an increase in the GFR. Based on Brenner's theory, hyperfiltration can cause an increased GFR and indicates renal injury in some clinical conditions. Glomerulus hyperfiltration can result from peripheral vascular changes, such as arterial stiffness and endothelial dysfunction.[15,16]

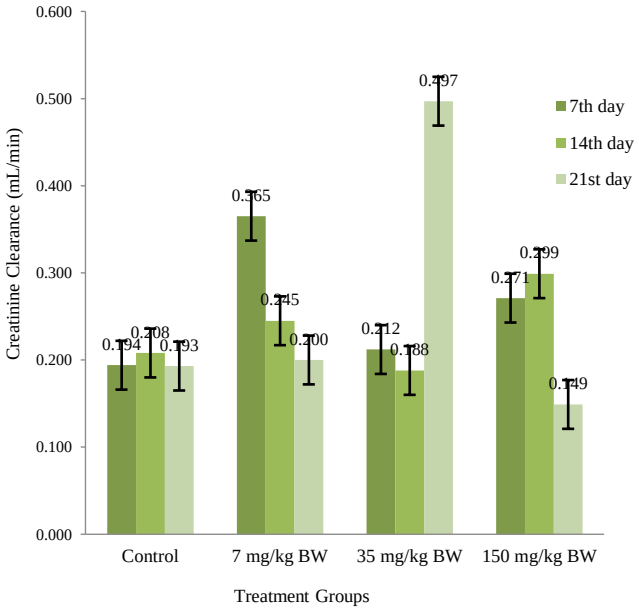

Figure 5: The effects of dose and duration of administration of *Hydrocotyle sibthorpioides* Lam extract on creatinine clearance in male Wistar rats.

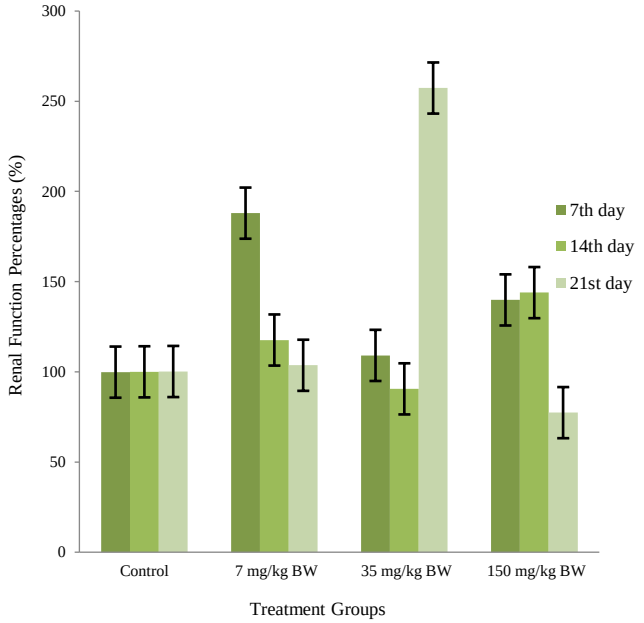

Figure 6: The effects of dose and duration of *Hydrocotyle sibthorpioides* Lam extract administration on the percentage of renal function in male Wistar rats.

The present study shows that almost all statistically tested parameters were influenced by a variation in dose but not in the duration of administration, as indicated by a wide margin of standard error. However, the percentage of renal function showed a significant increase (>100%) compared to the control, indicating possible renal impairment. The diuretic activity of *Hydrocotyle sibthorpioides* led to a high percentage of renal injury, likely due to flavonoids present in the ethanol extract.[13]Many flavonoids have diuretic activity, which increased urine volume by increasing the GFR.[14]

Based on Brenner's theory, an increased GFR, caused by hyperfiltration can lead to renal injury, particularly if the hyperfiltration is prolonged.[15,16] The present study showed that the percentage of renal function was significantly affected by doses of 7 and 35 mg/kg BW of the extract but not by doses of 150 mg/kg BW.

Conclusion

Various concentrations of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) extract affected renal creatinine clearance. The duration of administration of *Hydrocotyle sibthorpioides* extract did not appear to affect creatinine clearance.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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