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Original Article

Hepatoprotective and Nephroprotective Activity of *Artemisia absinthium* L. on Diclofenac-induced Toxicity in Rats

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ABSTRACT

Background: *Artemisia absinthium* L. is known for its antimalarial activity however, hepatoprotective activity of aqueous extracts has also been reported but, nephroprotective activity not yet evaluated. **Objective:** To evaluate the hepatoprotective and nephroprotective activities of *A. absinthium* against diclofenac-induced toxicity on rats. **Materials and Methods:** Three different doses of methanol and ethyl acetate extract of *A. absinthium* (50, 100 and 200 mg/kg/day) were evaluated and compared with silymarin 100 mg/kg. Rats received these doses for 5 days and on the 3rd and 4th day diclofenac (50 mg/kg i.p.) was administered 1 h after treatment. Animals were sacrificed 48 h after the last injection of diclofenac. Biochemical blood parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea and creatinine, and histopathologic changes of liver and kidney were studied and evaluated. **Results:** *A. absinthium* reduced the elevated blood levels of ALT, AST, ALP, urea and creatinine with the methanol extract to 200 mg/kg/day being more effective. The histopathologic evaluation suggested that *A. absinthium* decreased hepatic and renal necrosis induced by diclofenac. **Conclusions:** Hepatoprotective and nephroprotective activities of methanol and ethyl acetate extract of *A. absinthium* were demonstrated, being methanol extract to 200 mg/kg/day the most effective. This provides scientific support for the use of medicinal plants such as *A. absinthium* in the treatment of liver and kidney disorders.

Key words: *Artemisia absinthium*, Diclofenac, Hepatoprotective, Nephroprotective, Biochemical parameters, Histopathology.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed groups of drugs around the world[1], with diclofenac being the most widely used NSAIDs to treat pain in musculoskeletal, rheumatoid and osteoarthritis injuries.[2] It is accepted that the mechanism of action of all NSAIDs is the inhibition of cyclooxygenase (COX) producing a decrease in the synthesis of prostanoids.[1,3] In addition to the processes associated with inflammation, many physiological biochemical processes are affected by prostanoids, such as gastric mucus secretion and hemodynamics.[3] This is the reason why NSAIDs, such as diclofenac, can lead to adverse effects on the gastrointestinal tract and the kidney, where the latter is partly due to reduced renal blood flow causing ischemia and subsequent necrosis.[4] Although some studies mention that the damage caused to the kidney would not be due to renal portal vasoconstriction, but rather due to an increase in reactive oxygen species (ROS)[1,2,5], a mechanism also involved in liver damage caused by this drug, which oxidative stress, activation of cytochrome P450 and transition of mitochondrial permeability are added to produce this damage at the liver level.[6]

Both the liver and kidney play important roles in the elimination of waste produced by the organism.[1,7] On the one hand, liver damage is associated with loss of several important metabolic functions[8], while the accumulation of toxins in the kidneys would cause other complications in the body.[9] In the absence of reliable hepatoprotective drugs in conventional medicine[7], which in turn has protective activity at the kidney level, it is necessary to look towards medicinal plants where those with hepatoprotective properties have been sought over the years[10-12], *Artemisia absinthium* L. (*A. absinthium*) being one of the plants investigated due to this property[13-15]. In addition, flavonoids, terpenoids and coumarins was reported like responsables of pharmacological activities; and being flavonoids, possible responsible for hepatoprotective activity.[14,15]

A. absinthium (Figure 1), and the genus *Artemisia*, is known for its antimalarial activity[15], however, antibacterial[16], neuroprotective[17], hepatoprotective[18], antiulcer[19], antitumor[20], antidepressant[21] and antioxidant[22] activity has also been reported, nephroprotective activity not yet evaluated. The hepatoprotective activity of aqueous extracts of *A. absinthium* has been proven against carbon tetrachloride toxicity, suggesting that the activity is due to the antioxidant effect it has[23], and the efficacy of alcoholic extracts has also been proven[14,15]; however, lipophilic metabolites could also be involved in a greater hepatoprotective and nephroprotective activity[10,11], so in this study the hepatoprotective and

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Figure 1: *Artemisia absinthium* L.

nephroprotective activity of methanolic and ethyl acetate extracts of *A. absinthium* on diclofenac-induced toxicity in rats was evaluated.

MATERIALS AND METHODS

Vegetal material

The fresh leaves of *A. absinthium* were collected from Contumazá (Cajamarca, Peru) in April 2019. The specimens were admitted to the *Herbarium Truxillense* (HUT) of the Universidad Nacional de Trujillo, Trujillo, Peru with the code 59573.

Preparation of the extract

The leaves were washed with water, dried in the environment and homogenized to fine powder to be stored in amber glass containers. Two hundred grams of fine powder were macerated with methanol and ethyl acetate at room temperature for seven days with occasional stirring. The macerates were sterile filtered in Biological Safety Cabinet and then dried to be stored at -10 °C. For the process, the dry methanolic (ESM) and ethyl acetate (ESA) extracts were dissolved in sterile water at the desired concentration.

Chemicals

Drugs such as diclofenac and silymarin were obtained from commercial pharmaceuticals: Diclofenac Genfar® and Silymarin Genfar®; on the other hand, the chemical reagents were of analytical reagent grade and were obtained from the following sources; ethyl acetate (Emsure®), methanol (Emplura®), formaldehyde 37% (Merck), sodium chloride 9% (Medifarma).

Animals

Two and a half months old Holtzman albino rats of both sexes (220-250 g) were used for this investigation. The animals were obtained from the Instituto Nacional de Salud (INS). All rats were kept in standard plastic rat cages and wood shavings were used as bedding. The animals were kept in the bioterium of the Faculty of Pharmacy and Biochemistry, National University of Trujillo. Animals were enabled with standard photoperiod environmental condition (12:12 h dark: light cycle) and temperature [(25 ± 2) ° C] They were provided with commercial food for rats and mice (Food purchased from INS) and water administered *ad libitum*. This study was approved by the Norbert Wiener University Ethics Committee under opinion N° 006-05-2020 FB/UPNW.

Hepato- and nephro-protective evaluation

Rats were divided into nine groups with six rats in each group. Group I (control) received the vehicle alone (sterile water 10 mL/kg of body weight p.o.) for 5 days. Group II (diclofenac control) received diclofenac (50 mg/kg i.p.) on the third and fourth day. Groups III (ESM-50), IV (ESM-100) and V (ESM-200) were treated with ESM at the dose of 50 mg/kg, 100 mg/kg and 200 mg / kg of body weight p.o. per day, respectively, for 5 days, and groups VI (ESA-50), VII (ESA-100) and VIII (ESA-200) were treated with ESA at the dose of 50 mg/kg, 100 mg/kg and 200 mg/kg of body weight po per day, respectively, for 5 days and on the 3rd and 4th day diclofenac (50 mg/kg i.p.) was administered to the six groups 1 h after treatment of the extract. Group IX (standard) was treated with the standard medicine silymarin (100 mg/kg p.o.) for 5 days and on the third- and fourth-day diclofenac (50 mg/kg i.p.) was administered 1 h after medical treatment. Animals were sacrificed 48 h after the last injection of diclofenac under mild anesthesia with Ketamine 10%. The blood, kidney and liver of each animal were collected.

Estimation of biochemical parameters

The separated serum was used to determine biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) creatinine and urea. The absorbance of all parameters was measured on a Persee T7 UV-VIS spectrophotometer.

Histopathological study

The liver and kidney of each animal were kept in formaldehyde at 10% for 8 days before being analyzed. A portion of the organ was cut to approximately 6 mm in size and fixed in a phosphate buffered formaldehyde solution at 10%. Samples were dehydrated through graduated alcohol, clarified with xylene, and embedded in paraffin. After being embedded in paraffin wax, 5 µm thick sections of tissue were cut and stained with hematoxylin-eosin. Thin sections of tissue were converted to permanent slides and submitted for histopathological analysis by a veterinary pathologist.

Statistical analysis

The data obtained from the animal experiment are expressed as mean ± SD. The charts were prepared using Microsoft Excel® and the data were subjected to an analysis of variance (ANOVA) followed by the *post hoc* Tukey test. Values are considered statistically significant at $P < 0.05$.

RESULTS

Serum biochemical analysis

The comparison of the biochemical parameters is shown in Figure 1. The levels of AST, ALT, ALP, urea and creatinine in the group treated with diclofenac sodium (Control Diclofenac) show significant elevation compared to the Control group ($P < 0.05$). AST, ALT and ALP levels determined liver function and urea and creatinine, renal function.

The groups that received treatment with extracts of *A. absinthium* significantly decreased biochemical parameters compared to the diclofenac control group. Extracts ESM-50, ESM-100 and ESM-200 show significant decrease in the five parameters compared to diclofenac control group. ESM-50 shows similar decrease in ALP with silymarin group (IX). ESM-100 significantly lowers AST levels, it also lowers ALT levels as much as ESA-100 and urea and creatinine levels to normal like levels. ESA-200 decreases ALT and urea levels to control like levels ($P < 0.05$). The ESA-50, ESA-100 and ESA-200 extracts significantly decreased biochemical parameters compared to the diclofenac control group, however, the decrease in ESM was greater.

Histopathological changes

Liver and kidney lesions decreased mostly with the administration of ESM (Figure 2) compared to ESA (Figure 3), showing a greater protective effect by decreasing degeneration and necrosis. The protective effect was also observed in the group treated with silymarin (Figure 4). The diclofenac control group revealed liver lesions characterized by hepatocytes in a degenerative and necrosis state, condensation of chromatin or picnosis, and kidney lesions, showing the vast majority of dilated renal tubules, cubic epithelium in hydropic degeneration and necrosis state, and marked distortion of the renal glomerulus. (Figure 4). On the other hand, in the normal control, the histological sections of the animal liver and kidney showed normal cells (Figure 4). This agrees with the results obtained from the biochemical parameters.

In relation to the treatment with methanolic extract, the following results are described, Figure 2: (A) Rat liver. Diclofenac induction. Distorted liver cord arrangement (**inset**) relative to central vein (**CV**). Necrosis (**n+**) of hepatocytes and fatty change (**arrow**) because of NSAIDs, with a slight effect of the dose of *A. absinthium*. (B) Rat liver. Diclofenac induction. There is still a disorder in the arrangement of the hepatic cords towards the central vein (**CV**). There is still necrosis (**n+**) of hepatocytes and decreased fat change (**arrow**) because of NSAIDs. An improvement in liver architecture is evidenced by the effect of the higher dose of *A. absinthium*. (C) Rat liver. Diclofenac induction. Disposition and arrangement of the hepatic cords radially towards the central vein (**VC**). Few necrotic hepatocytes (**n+**), marked sinusoidal dilation (**sh**) and decreased fat change (**arrow**), changes attributable to a higher dose of *A. absinthium*. (D) Rat kidney. Diclofenac induction. Mostly dilated tubules (**dt**). Necrosis of the tubular epithelium (**n+**) attributable to the toxic effect of the NSAID is still evident. (E) Rat kidney. Diclofenac induction. Tubular dilation (**dt**), decrease in tubular epithelial necrosis and presence of slight mononuclear infiltration (**arrow**) attributable to the effect of NSAIDs. The histological picture is better in response to the higher dose of A. absinthium. (F) Rat kidney. Diclofenac induction. The renal tubules (**tr**), maintain their contour and cellular activity, little epithelial necrosis. Normal renal glomerulus (**GR**).

In relation to treatment with ethyl acetate extract, the following results are described, Figure 3: (A) Rat liver. Diclofenac induction. Presence of hepatocytes in necrosis (**n+**), fat change (**arrow**) and notorious sinusoidal dilation (**sh**). Hepatocyte cords arranged radially to the central vein (**CV**), marked pigment infiltration (*) attributable to *A. absinthium*. (B) Rat liver. Diclofenac induction. Necrosis (**n+**), sinusoid dilation (**sh**). Hepatocyte cords converge towards the central vein (**CV**), infiltration of bile pigments (*) and recovery of the change in hepatocytes due to probable effect on *A. absinthium*. (C) Rat liver. Diclofenac induction. Decreased necrosis (**n+**), sinusoidal dilation (**sh**). Pigment infiltration is maintained (*), few hepatocytes with fatty degeneration (**arrow**) due to a probable effect at a higher dose of *A. absinthium* (D) Rat kidney. Diclofenac induction. Renal tubules dilated and looking swollen from hydropic swelling (**tr**). Necrosis of cubic cells of the tubular epithelium

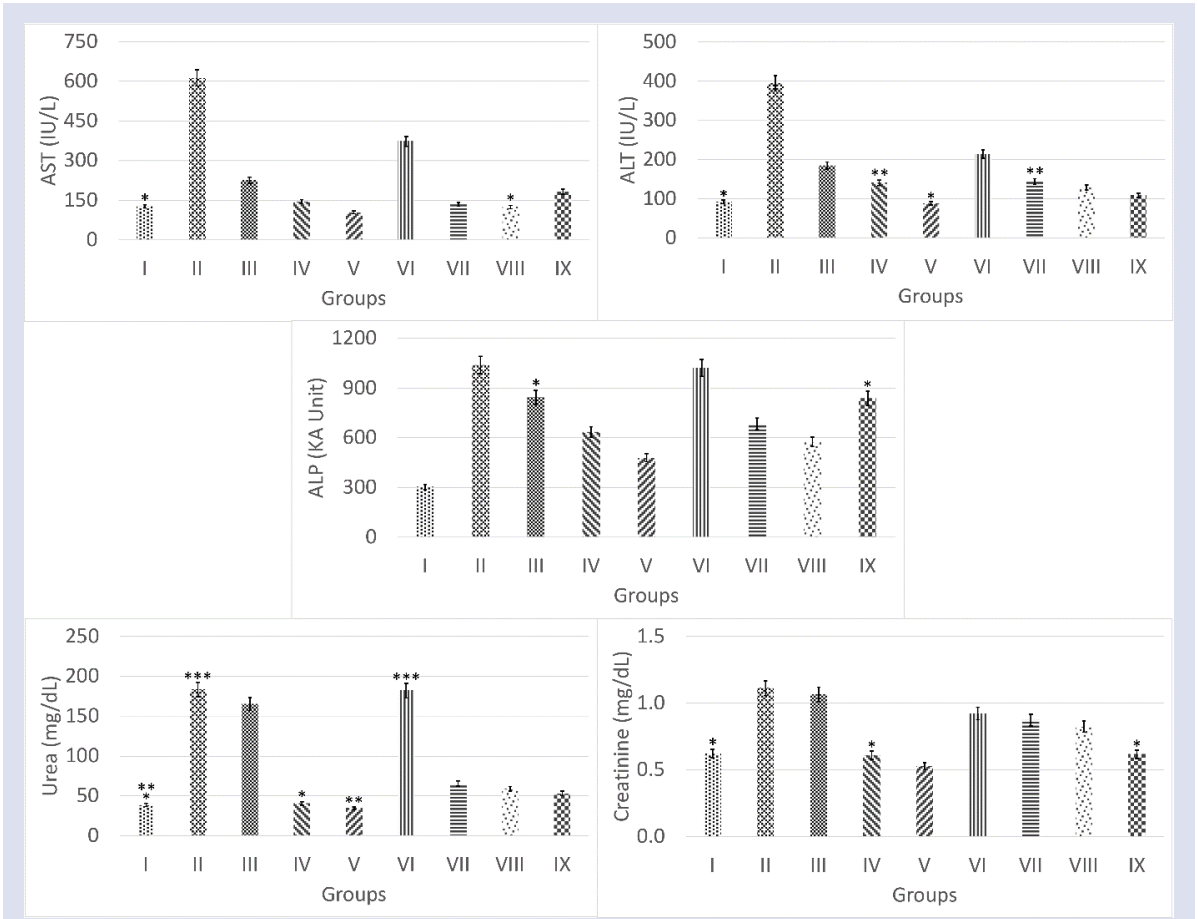

Figure 2: Effect of A. absinthium extracts on biochemical parameters in diclofenac-induced liver and kidney damage in rats. Group I: Control. Group II: Diclofenac Control. Group III: ESM-50 mg/kg + diclofenac. Group IV: ESM-100 mg/kg + diclofenac. Group V: ESM-200 mg/kg + diclofenac. Group VI: ESA-50 mg/kg + diclofenac. Group VII: ESA-100 mg/kg + diclofenac. Group VIII: ESA-200 mg/kg + diclofenac. Group IX: Silymarin 100 mg/kg + diclofenac (Standard). Results expressed as mean ± SD (n = 6). *, ** and ***: Statistically similar groups according to ANOVA statistical analysis and post hoc Tukey test at P <0.05.

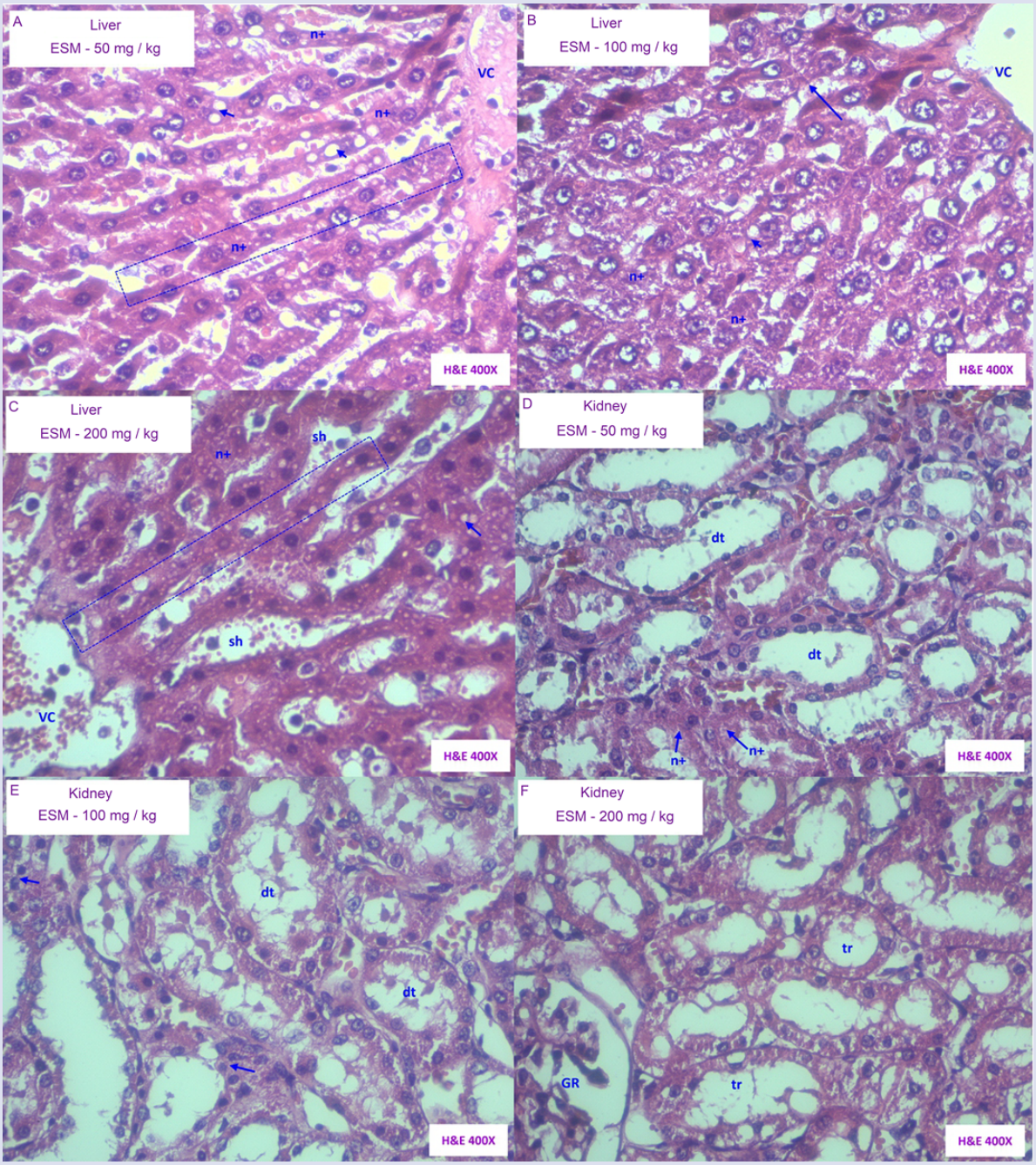

Figure 3: Photographs of liver and kidney sections from treatment with *A. absinthium* methanolic extract (Hematoxylin and eosin stained, 400X).

(**n+**). Distorted renal glomerulus (**GR**), changes attributable to the toxic effect of NSAIDs. The dose of *A. absinthium* (50 mg) is insufficient. (E) Rat kidney. Diclofenac induction. Swollen renal tubules (**tr**). Necrosis in tubular epithelium (**n+**). Renal glomerulus (**GR**) in apparent atrophy. No effect of *A. absinthium* supply is seen. (F) Rat kidney. Diclofenac induction. Swollen renal tubules (**tr**). Necrosis in tubular epithelium (**n+**). Renal glomerulus (**GR**) in apparent atrophy. No effect of *A. absinthium* supply is seen.

In relation to the results observed in the diclofenac, standard and control groups, the following is described, Figure 4: (A) Liver. Diclofenac control. Radially to the central vein (**CV**) the hepatocyte cords flow, many of them in a degenerative state and necrosis (**arrows**), chromatin condensation or picnosis (+) is observed. The aspect is progressive and corresponds to a lesion caused by diclofenac. (B) Rat kidney. Diclofenac control. Many dilated renal tubules, hydropically degenerating cubic epithelium and necrosis (**arrows**) and marked distortion of the renal glomerulus (**RG**) as a result of the toxic effect of NSAIDs. (C) Liver. Standard group. Towards the central vein (**CV**) the hepatocyte cords show recovery of their vitality, however, there are still liver cells in a degenerative to necrotic state (**arrows**). The behavior of silymarin has a positive effect that would be subject to the dose and time of treatment. (D) Rat kidney. Standard group. Dilation and swelling of the renal epithelium with decreased necrosis are maintained (**arrows**). The renal glomerulus shows recovery (**GR**) attributable to the effect of silymarin. (E) Liver. Control group. Radially to the central vein (**CV**), the hepatocyte cords flow, showing their contour and nucleus well-dyed corresponding to normal tissue. (F) Rat kidney. Control group.

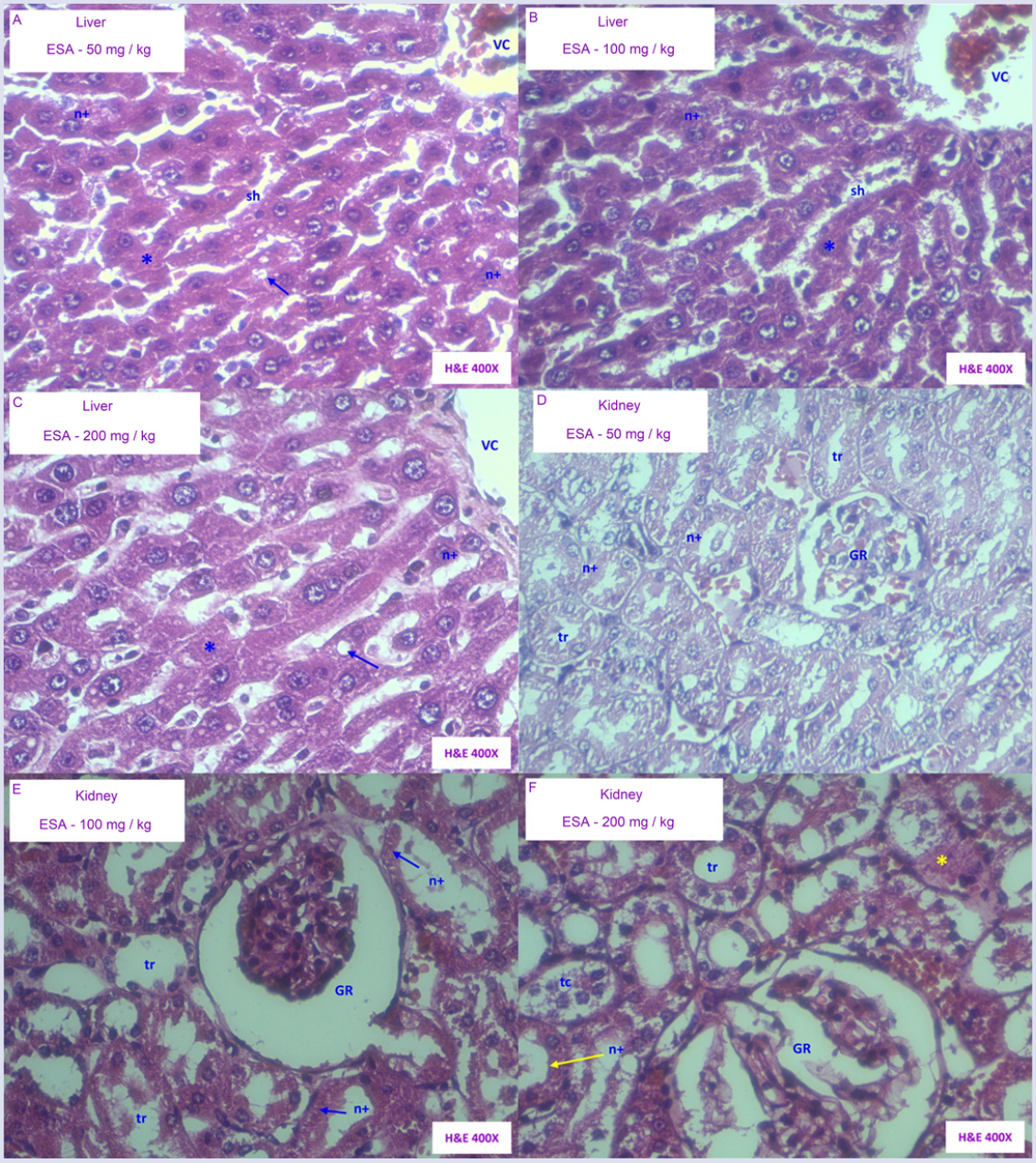

Figure 4: Photographs of liver and kidney sections from treatment with ethyl acetate extract of *A. absinthium* (Hematoxylin and eosin stained, 400X).

The histological panorama corresponds to a normal stage of the renal cortex, a normal glomerulus (**GR**) and slight dilation of the tubules with maintenance of their cubic epithelium (**arrows**).

DISCUSSION

Both liver and kidney damage are adverse effects caused by NSAIDs, diclofenac being one of the drugs with the highest probability of damage in this drug group.[4,24] Metabolically, diclofenac is mainly eliminated as its 4-hydroxylated metabolite in humans, whereas in rats the acyl glucuronide (GA) pathway predominates[25]; The AG metabolite of the drug has been shown to be capable of covalently modifying cellular proteins, and covalently binding to liver proteins in rats dependent on the activity of the multi-drug resistance protein 2 (hepatic canalicular transporter).[26] Therefore, liver toxicity has been classified as idiosyncratic. On the other hand, diclofenac-induced renal toxicity is caused by the attack on renal mitochondria, leading to the production of ROS, causing apoptosis and DNA damage.[1,5]

Diclofenac administered in rats increased AST, ALT, and ALP levels, indicating liver damage[27], and in turn increased creatinine and urea, indicating kidney damage.[28] The increase in these values, as well as the damage to liver and kidney tissue, decreased with the pretreatment of the dose-dependent *A. absinthium* extracts, the treatment with methanolic extract being more effective than with ethyl acetate extract. The hepatoprotective potential of the hydroalcoholic and aqueous extract of *A. absinthium* has been previously tested[13-15,17,23] and this study corroborates the potency of the methanolic extract against an ethyl acetate extract from the same plant; previous works demonstrated the hepatoprotective effect against CCl_4 and non-diclofenac, a medicine frequently used against pain and inflammation[29], and the activity observed in this research is similar to that reported by Mohammadian

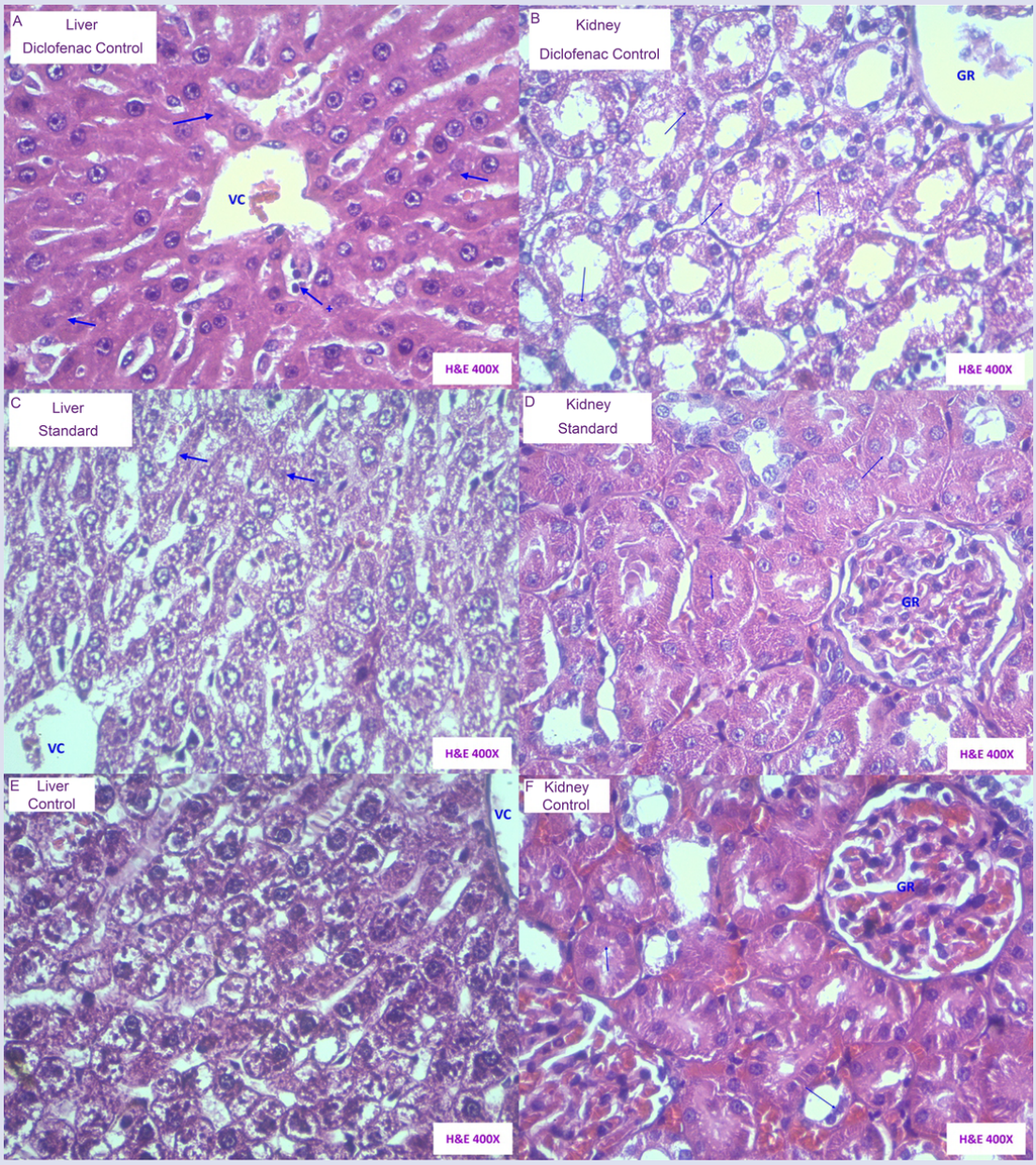

Figure 5: Photographs of liver and kidney sections of diclofenac control, standard and control (Hematoxylin and eosin stained, 400X).

et al[15] and Saxena *et al*[13]; Histopathological sections show the recovery effect of hepatocytes with the production of bile juices induced by the supply of *A. absinthium*, which could counteract diclofenac-induced damage. Probable mechanism of hepato-protection by *A. absinthium* is on the liver antioxidant status. Liver is a organ involved in ROS generation and, in this study, antioxidant activities could have a important role in this protection.[15]

Furthermore, the nephroprotective effect of *A. absinthium* has not been previously studied, and the greater effect of methanolic extract has been demonstrated by this study. Although the nephroprotective effect of *A. absinthium* has not been previously studied, the effect of other *Artemisia* species has been studied, showing the effect of *Artemisia arborescens*[30], *Artemisia herba-alba*[31], *Artemisia sieberi*[12], *Artemisia campestris* L.[32] and *Artemisia scoparia*[33], being similar to the one presented by *A. absinthium*. At the histopathological level, the methanolic extract of *A. absinthium* showed a substantial decrease in tubular and glomerular damage, becoming physiologically almost normal. This decrease in the above alterations can be attributed to the antioxidant properties of *A. absinthium*.[21] Supplementation with methanolic extract of *A. absinthium* can help preserve the antioxidant / free radical ratio by improving the elimination of free radicals by antioxidant enzymes, which reduces these previous alterations; obviously this could demonstrate the protective role in the kidney injury produced by diclofenac[34,35], as well as the hepatoprotective effect as both effects are greater in the methanolic extract at doses of 200 mg/kg/day. The hepatoprotective activity and the nephroprotective activity of an extract is a dose-dependent activity[36].

Hepatoprotective activity was similar to *Artemisia Herba-alba*[37] and *Artemisia sieberi*[38], it was better than *Artemisia arborescens* essential oil[39] and *Artemisia campestris*[40], and not than effective such *Artemisia scoparia*[41]. In *Artemisia annua*, hepatoprotective activity is connected to their content of hydroxycinnamoyl quinic acids and flavonoids.[42]

An important mechanism of hepatoprotective activity is related to its ability to transfer hydrogen to free radicals, activate antioxidant

enzymes, and inhibit oxidases[43]. Methanol is the solvent capable of extracting the greatest amount of phenolic compounds[44], a type of compound that presents a high antioxidant capacity that is present in *A. absinthium*[45], however, bioguided phytochemical prospecting is required to determine the metabolite(s) responsible for this activity and its subsequent determination of its mechanism of action by which it exerts its pharmacological action.

CONCLUSION

In conclusion, both methanolic extract and ethyl acetate extract of *A. absinthium* were effective in preventing kidney and liver damage caused by diclofenac in rats, being the most effective methanolic extract. However, the hepato- and nephro-protective mechanisms have not yet been determined, although antioxidant effects may be involved.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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AUTHORS' CONTRIBUTIONS

VEVLT produced the first draft. CSC performed organ harvesting for histopathological analysis. ACP kept the animals during the investigation and administered treatments together with VEVLT, CSC and CAV. CAV performed the statistical analysis and the preparation of images. WSG collected the plant species, entered the herbarium and corrected the article. CGS made article corrections and made substantial additions. GRR carried out the preparation of extracts. LCR and JCF did blood collection and biochemical analysis.

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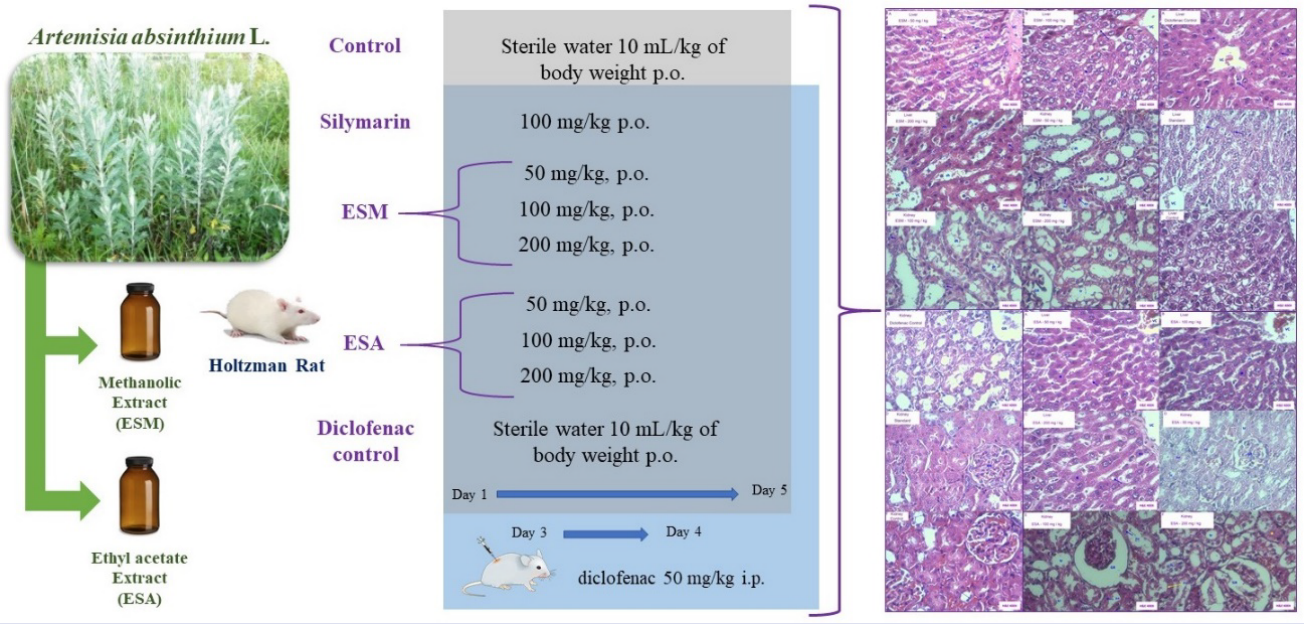
GRAPHICAL ABSTRACT



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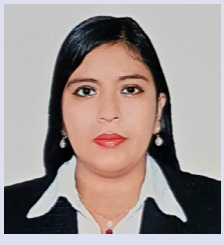

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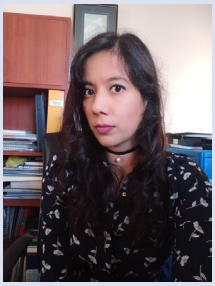

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