

Research Article

Sub Chronic Toxicity Study of Aqueous Leaves Extract of *Maytenus Gracilipes* on Some Biochemical Parameters and Histopathology of Liver and Kidneys in Mice

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Abstract

Introduction: Traditional medicine is an ancient medical practice that is still widely used in prevention and treatment of various health problems worldwide, including Ethiopia. *M. gracilipes* is one of medicinal plant in Ethiopia used for treatment of various ailments still is very common. This study evaluated the sub-chronic toxic effects of *M. gracilipes* aqueous leaves extract on biochemical parameters and histopathology of liver and kidneys. **Methods:** For sub-chronic toxicity study a total of 30 mice were used, three groups (I–III) of mice (10 animals each) were used. Group I served as control and received a vehicle while groups II and III daily administered with 700 and 2100 mg/kg extract respectively orally by using oral gavage for 90 days. At the end of the experiment, the mice were sacrificed by using diethyl ether; blood was collected for assessing biochemical parameters and histopathological evaluations on liver and kidneys were performed. **Results:** Sub chronic treatment of extract for 90 days, at 700 and 2100 mg/kg body weight did not induce any sign of illness and /or death and had no adverse effect on biochemical parameters and blood parameters. Liver and kidney sections also revealed normal architecture, except some pyknotic nuclei and focal mononuclear leukocytic infiltrations observed in some of the liver and kidney tissues at higher dose (2100mg/kg). **Conclusion:** The results of this sub chronic toxicity study showed that *M. gracilipes* aqueous leaves extract is safe at daily doses of 700 mg/kg body weight, even when taken for longer period. At higher doses, however, the extract may induce mild hepatorenal toxicity.

Keywords

Aqueous Leaves Extract, Sub-Chronic Toxicity, Histopathology, *Maytenus Gracilipes*, Liver and Kidneys

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1. Introduction

Herbal medicine is also known as phytochemical or botanical medicine. According to World Health Organization (WHO), herbal medicine includes 'herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredient parts of plants or other plant materials or combinations there [1]. Herbal medicine is generally considered as an integral part of dietary supplement. There is a growing interest in herbal medicine due to its long history of application and general belief that herbs are natural and intrinsically safe.

Traditional medicine is an ancient medical practice that is still widely used in prevention and treatment of various health problems worldwide, including Ethiopia [2-5]. There is a growing recognition that knowledge of traditional medicine is important not only for its potential as therapeutic drugs but also for its socioeconomic and cultural components [6-8]. Before the development of synthetic or semi-synthetic medicines, folklore use of herbals was very common in rural areas, the use of herbal preparations for the treatment of various ailments is still very common [9]. Many of the modern pharmaceuticals are derived from medicinal herbs. In the eighteenth century, when the medicinal therapy era was being introduced, the herbal treatment was the most preferred and available therapy. Many compounds from herbal origin have achieved widespread use as medicinal agents e.g. Taxols from *Taxus baccata* (English yew) as anticancer agents, Silibinin from *Silybum marianum* (Milk thistle) as liver tonic [10, 11]. The herbal therapy encompasses Ayurvedic, Naturopathic, Biochemical, Unani, Chinese, African and Native American medicine [12]. Herbal medicines have attained the widespread acceptability as natural therapeutic agents for various diseases like diabetes, arthritis, renal and liver diseases, obesity and cardiovascular disorders [13].

The ethnomedical value of *Maytenus gracilipes* is numerous such as antioxidant, anti-inflammatory, anti-ulcer, anti-cancer and many stomach complications, antitumor, antimicrobial [14].

2. Methods

2.1. Plant Collection

The leaves of *Maytenus gracilipes* were collected from Ankober woreda, near Atsie Minilik loge, about 42 km away from Debre Birhan, Northern Shawa, Amhara Region, 130 km North of Addis Ababa during the month of May 2016. Specimens of the plant were identified by a taxonomist and samples were deposited at the National Herbarium in the College of Natural and Computational Sciences, Addis Ababa University (AAU) for future reference with a Voucher specimen number (119/AMA/PHARM). The collected plant material was processed and extracted at phytochemical laboratory of Ethiopian Public Health Institution (EPHI). The leaves of plant were dried under shade area at room temperature and crushed, and dry powder was prepared for the study.

2.1.1. Preparation of Aqueous Leaf Extracts of *Maytenus Gracilipes*

The powdered leaves were macerated with distilled water for 72 hrs with intermittent agitation by orbital shaker DS-500. Then, the supernatant part of agitated materials was separated from the undissolved portion of the plant material. The supernatant portion was filtered with 0.1 mm² mesh gauze and then with Whatman grade 1 filter paper with pore size of 11 µm. The filtrate was then concentrated by evaporating the solvent using a rotary evaporator (BUCHI Rota-vapor type R-205, Switzerland) under reduced pressure at a temperature of 40–45 °C. Then the residue was dried by steam bath at 40 °C for period of one week to make it dry [15].

2.1.2. Preparation and Grouping of Experimental Animals

The healthy and non-pregnant young Swiss albino mice aged 8–10 weeks and weighing 25–30 gm of both sexes were obtained from EHPI, Addis Ababa. The animals were acclimated to laboratory conditions for 5 days. They were housed in standard cages and kept under standard condition at a temperature of (21±2 °C) with 12 hrs light/12 hrs dark cycle and were provided with free access to standard diet and tap water *ad libitum* according to OECD guideline [16].

Animals were randomly assigned to a control and two treatment groups. Each animal was assigned a unique identification number. A total of 30 mice of both sex containing 10 mice per group (five female and five male) were used for sub chronic toxicity study.

2.2. Sub Chronic Toxicity Study

The sub chronic toxicity study was conducted for 90 days to evaluate the toxic effect of the extract on some biochemical parameters and histopathology of liver and kidneys by using some modification of the method used by a study [17].

For this study healthy adult mice of both sexes were used. Thirty mice were randomly distributed into three groups (I, II, and III) each consisting of ten mice (five female and five male) per group. Groups II and III were orally administered with aqueous extract of leaves at doses of 700 and 2100 mg/kg body weight per day respectively, for 90 days using oral gavage. Group I served as control group and received distilled water. Clinical observation was carried out for 90 days and their weight was measured weekly for thirteen weeks. On the 90th day the final weight of the mice was measured and then they were anesthetized under diethyl ether and blood samples were collected from each animal by cardiac puncture.

2.3. Blood Collection for Biochemical Analyses

Blood samples in the test tubes without anticoagulant could clot and sera were obtained by centrifuging the blood using an electrical centrifuge (HUMAX-K, HUMAN-Germany) from which blood chemistry (Urea, Creatinine, Total Bilirubin, ALT, and AST) was studied to test renal and hepatic functions. Values in the sera were analyzed using Automated Clinical Chemistry Analyzer (AUTO LAB 18, clinical chemistry analyzer, Italy)

2.4. Target Organ Collection

After collection of blood samples, the mice were sacrificed by cervical dislocation and parts of the liver and the kidneys were dissected out; and gross pathological observation was performed on liver and kidneys to check for any gross lesions.

2.5. Histopathological Studies

The liver and kidney sections taken randomly for tissue processing were fixed in 10% neutral buffered formalin (NBF) overnight at room temperature. After fixation, the tissue sections were washed with water to remove excess fixatives for about six hours and dehydrated with increased concentration of alcohol of 70% for two hours, 90% for two hours, absolute alcohol-I, II for one and half-hours, and III overnight. The dehydrated tissues were cleared in two changes of xylene-for one and half hours and two and half hours. The tissues were then infiltrated with three changes of paraffin wax-for one and half hours, two and half-hours, and overnight. Finally, the tissues were embedded in paraffin wax in square metal plates forming tissue blocks, and then each tissue block was labeled and stored at room temperature till sectioned. The tissue blocks were sectioned in ribbons at a thickness of 5 μ m with Leica microtome (Leica RM 2125RT Nussloch GmbH, Germany). The ribbons of the section were collected at every 5th sections and put onto the surface of a warm water bath of temperature of 40 °C. The floating ribbons over the surface of warm water were mounted onto pre cleaned slides spread with egg albumin. The slides containing paraffin wax were arranged within the slide holder and placed in an oven with temperature of 40 °C for about 20 minutes so as to fix the tissue to the slides and allowed to cool at room temperature for 30 minutes and stained regressively with routine Harrisheamatoxylin for 6 minutes and then eosin for 17-20 second (H and E). For routine H and E staining, two series of coupling jars were prepared: one for paraffin removal and hydration and the other for dehydration and clearing. So, sections were placed in xylene I for 5 minutes and xylene II for 2 minutes again to remove the paraffin from tissue and

hydrated with decreasing concentrations of absolute I, II and 95% alcohol for two minutes each, 70% of alcohol for three minutes, and 50% alcohol for five minutes. The tissue sections were washed with tap water for five minutes and stained regressively with Harris heamatoxylin for 6 minutes and then washed under running tap water for five minutes again. The slides were immersed in acidic alcohol for differentiation and controlling over stained heamatoxylin for 1 second and then put in bluing solution (sodium bicarbonate) until they became blue. After bluing, the slides were counter stained with eosin for 17-20 seconds and then washed in tap water for two minutes. The sections were dehydrated with increasing alcohol concentration of 50%, 70%, 95%, absolute I and II for two minutes each. The dehydrated sections were cleared with xylene I and xylene II for three minutes each and permanently mounted on microscopic slides using DPX and cover slips and then observed under light microscope for the investigations of any histological change, thereby the histology of the treated groups was compared with histology of the control group. After examination, photomicrographs of selected samples of liver and kidney section from both the treated and control mice were taken under a magnification of x20 objective using (EVOS XL, USA) automated built-in digital photo camera.

2.6. Statistical Analysis

Data were presented as mean \pm SEM with 95% confidence interval and analyzed by SPSS version 21. And one way ANOVA followed by *post hoc* test (t-test) was used for multiple comparisons of the mean differences and responses of different doses of extracts. The difference between groups with respect to variables under investigation was significant at P value of less than 0.05.

3. Results

3.1. Effects on the Behavior, Gross Pathology, Organ, and Body Weight

Throughout the study period no sign of toxicity and mortality was observed on treated mice, which received 700mg/kg and 2100 mg/kg. Gross observation of the liver and kidneys of the treated mice showed no significant changes compared with the control group.

3.2. Effects on Biochemical Parameters

In the sub chronic toxicity study, the biochemical parameters of the treated groups (700 mg/kg and 2100 mg/kg) were not significantly different from the control group (table 1).

Table 1. Effect of aqueous leaf extract of *M. gracilipes* on biochemical parameters during sub chronic toxicity study.

Biochemical parameter	Control Group (GI)	Treated Group	
		700 mg /kg (GII)	2100 mg/kg (GIII)
ALP	98.9 ±30	107.35 ±341(0.81)	118.5 ±8.3(0.71)
ASP	117 ±7.10	152.5 ±16.5(0.45)	144.5 ±13.75(0.92)
Total Bilirubin	1.475 ±0.45	1.925 ±1.01(0.61)	2.245 ±7.5(0.26)
Urea	46.4 ±165	48.61 ±7.10(0.71)	53.4 ±2.6(0.53)
Creatinine	0.665 ±0.2	0.715 ±0.245(0.63)	0.815 ±0.725(0.72)

Values are expressed as mean ± SEM, N=5

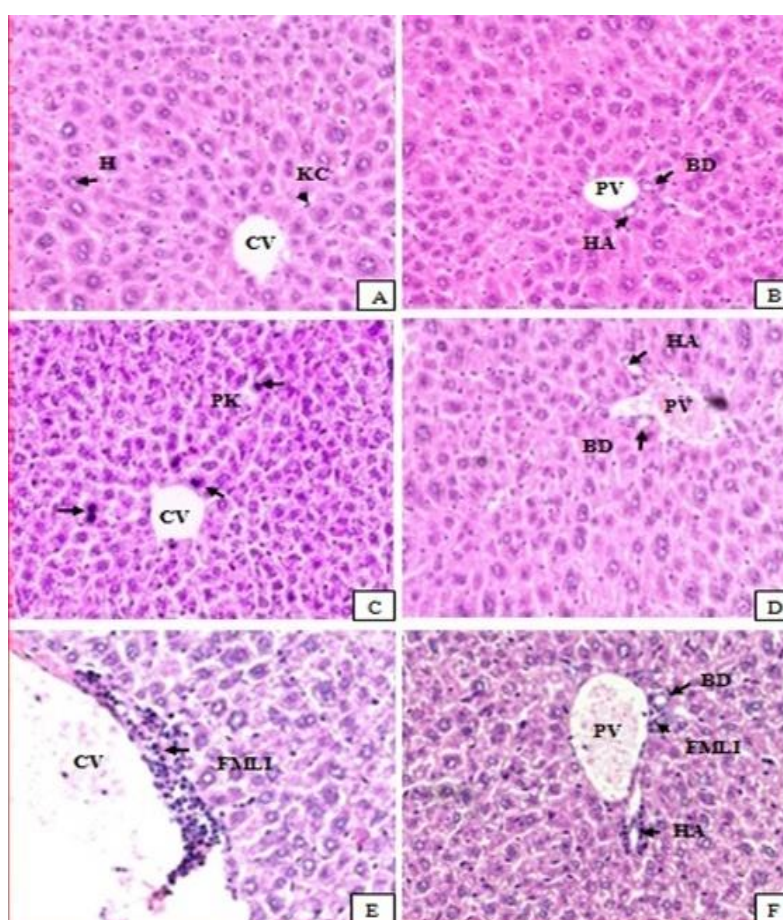


Figure 1. Photomicrographs of sections of liver from control mice (A & B) and mice treated with 700 mg/kg body weight/day (C & D) and 2100 mg/kg body weight/day (E & F). sections from female mice PK= pyknotic nucleus in hepatocytes in mice treated with 700mg/kg body weight/day (C); FMLI= focal mononuclear lymphocytic cellular infiltration in mice treated with 2100 mg/kg body weight/day (E&F) in central vein and portal area. H= Hepatocytes; BD=Bile duct; HA=Hepatic artery; CV= Central vein; PV=Portal vein; KC=kupfer cell. (Sections were stained with H&E, X300).

3.3. Effects on Histology of Liver

Histopathological studies of the liver sections in the control group (Figure 1(A) and 1(B)) showed normal appearance of central vein (CV) and hepatic sinusoids (S) lined by endothe-

lial cells (EC) with normal radiating hepatocytes. There was also normal appearance of the portal triad including hepatic portal vein (PV), interlobular bile duct (BD), and branches of hepatic artery (HA). Mice treated with aqueous leaf extracts of *M.gracilipes* at both doses of 700 mg/kg (Figure 1(C) and 1(D)) and 2100mg/kg (Figure 1(E) and 1(F)) showed normal

appearance of the central veins (CV) and hepatic sinusoids (S) lined with endothelial cells (E) with normal radiating hepatocytes. However, some pyknotic nucleuses in hepatocytes were exhibited and perivascular leukocytic cellular infiltration in the central and portal area **Figure 1(E) and (F)**.

3.4. Effects on Histology of Kidneys

Histopathological studies of the kidneys sections of mice treated with doses of 700 mg/kg (**Figure 2(C) and 2(D)**) and 2100 mg/kg (**Figure 2(E) and 2(F)**) showed no significant

microscopic/histological changes compared with the controls (**Figure 2(A) and 2(B)**). In the treated mice of kidney sections revealed normal glomerulus (G), Bowman's capsule lined with outer parietal layer/squamous cells (SC) and inner visceral layer/podocytes, urinary/bowman's space (BS), proximal convoluted tubules (PCTs) lined by simple cuboidal epithelium with brush border, distal convoluted tubules (DCTs) lined by simple cuboidal epithelium with more nuclei per cross-section, and macula densa (MD) with taller cells around the vascular pole.

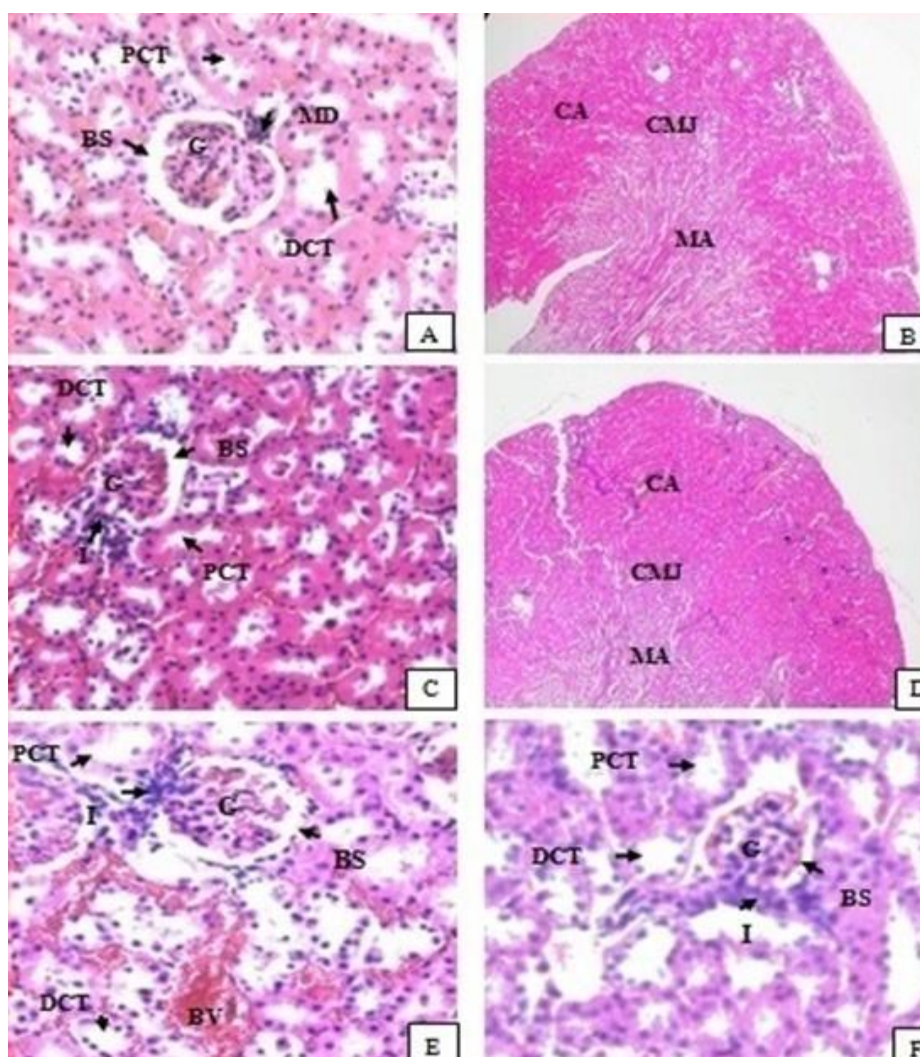


Figure 2. Photomicrographs of sections of kidney of control mice (A & B), mice treated with 700 mg/kg dose/day (C and D) and 2100 mg/kg dose body weight /day (E and F). sections from female mice Mononuclear lymphocytic cellular infiltration in mice treated with 700 mg/kg dose body weight (C), and treated with 2100 mg/kg dose body weight (E&F). DCT=Distal convoluted tubule; PCT=Proximal convoluted tubule; MD=Macula densa; BS=Bowman's space; G=Glomerulus; BV=Blood Vessels; CA=Cortical area; CMJ=Cortico medullary junction; MA=Medullary area; (Sections were stained with H & E, X300 for A, C, E & F X60 for B&D).

4. Discussion

In toxicological evaluation, biochemical parameters have significant roles as a marker because of their response to

clinical signs and symptoms produced by toxicants. Evaluation of hepatic and renal function is major importance to evaluate the toxic properties of extracts and drugs [18]. In the present study, all biochemical parameters did not show significant changes.

Measurements of urea and Creatinine levels in the blood

are usually performed to evaluate kidney function. Urea is the major nitrogen containing metabolic end product of protein catabolism, and Creatinine is a waste product of muscle energy metabolism. Creatinine and urea concentrations are used for the assessment of renal sufficiency.

Urea is usually increased in acute and chronic renal diseases. Urea clearance falls as the kidney fails and as a result, urea tends to accumulate with failed kidneys that are unable to excrete these substances at normal rate; this will raise blood urea level [19, 20]. In the present study, the mean values of urea have been shown with a slight increment at dose of 700 and 2100mg/kg with none significant with compare to the control. Creatinine is produced and released in to body fluids at a constant rate and its plasma concentration is maintained mainly by glomerular filtration. Consequently, both plasma concentration and its renal clearance have been used as markers of the glomerular filtration rate [19]. In the current study, the mean amount of Creatinine in treated groups showed slight increment but not significant compared to the control group.

Serum total bilirubin change is caused by a change in the volume of plasma water and a change in the concentration of one or more specific proteins in the plasma. Decrease in the volume of plasma water (hyperproteinemia) is noted in cases of dehydration due to inadequate water intake or excessive water loss, in case of severe vomiting or diarrhea [19]. In the current study, the amounts of total bilirubin were slightly increased at dose of 700 and 2100 mg/kg compared to control but not statistically significant.

The abnormal elevation of the liver enzymes (ALT and AST) is usually associated with liver damage or change in bile flow. ALT is found primarily in the liver and is the most sensitive marker for liver cell damage. When a cell is damaged, it leaks this enzyme into the blood. AST is found primarily in the red blood cells, cardiac and skeletal muscles, and kidney. AST is not specific to liver as ALT. In this study, the mean values of AST and ALT at dose 700mg/kg and 2100 mg/kg were slightly higher compared with control, but it was not significant. This was supported by the absence of histopathological changes in the liver of treated mice.

The liver and kidneys have fundamental roles in the metabolism and excretion of drugs or plant products. Plant chemicals and their metabolites might result in toxicity or cell damage on target organs [21, 22]. In the current histopathological examination of the liver, mice treated with doses of 700 mg/kg and 2100 mg/kg of the aqueous leaves of *M. gracilipes* showed no change in the microscopic structure of the liver. The general architecture of the liver, appearance of the hepatocytes, the hepatic sinusoids, portal triads, and central veins are normal as compared with controls. The result was also supported by the no adverse effects of the extract in any of the biochemical markers (such as ALT and AST), which showed statistically insignificant changes compared with control group [23]. This finding agreed with work of Amare [22] who reported that mice treated at a dose of 200 and 400

mg/kg bodyweight of the aqueous leaves extract of *S. guineense* showed no histopathological changes compared to control group, whereas the tissue morphology of mice treated with 600mg/kg showed hemorrhagic centrilobular necrosis and fatty cytoplasmic vacuolation of the hepatocytes.

In the histopathological study of the kidney, mice treated with both doses (700 and 2100mg/kg) of the extract showed no significant difference compared to controls. The sections of the kidneys of treated mice showed normal general structure of the kidney and the normal appearance of glomeruli and tubules. The proximal convoluted tubules, distal convoluted tubules, and macula densa are normal. The result was further supported by the values of biochemical parameters of the blood (such as Urea, Creatinine and total Bilirubin/protein), which are main indicators of kidney damage [23]. This was in line with the work of Amare [22]. Who reported absence of difference in tissue morphology between control group and mice treated with the low dose, 200 mg/kg.

5. Conclusion

Sub chronic toxicity study of the aqueous leaves extract of the *M.gracilipes* did not adversely affect the biochemical parameters of treated mice at both doses and there was no signs of toxicity observed in the histology of kidney and liver sections of treated mice.

Abbreviations

AAU: Addis Ababa University
 ALP: Alkaline Phosphatase
 ANOVA: Analysis of Variance
 ALT: Alanine Transaminase
 AST: Aspartate Transaminase
 DPX: Dibutyl Phthalate in Xylene
 EDTA: Ethylene Diamine Tetra-acetic Acid
 EPHI: Ethiopian Public Health Institute
 H & E: Haematoxylin and Eosinconcentration
 OECD: Organization of Economic Cooperationand Development
 SPSS: Statistical Package for Social Science
 SEM: standard Error of Mean
 WHO: World Health Organization

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Authors' Contribution

Mengistu Ayele and Minale Fekadie conceived and designed the study; performed the experiments; and analyzed the data. Mekebeb Afework, Eyasu Mekonnen, Asfaw De-bella, and Tesfaye Tolessa helped to design the study and supervised the study. Wondwossen Ergete helped to perform histopathological study. Mekebeb Afework and Eyasu Mekonnen drafted and edited the manuscript. All authors approved the final manuscript.

Data Availability

The data used to support the findings of this study are included within the manuscript.

Ethical Approval

Ethics approval for this study was obtained from the Scientific and Ethical Review Committee of the Department of Anatomy, Addis Ababa University.

Conflicts of Interest

The authors declare no conflicts of interest.

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