# EFFECT OF LOCAL ARAKE ON HEPATORENAL STRUCTURES AND FUNCTIONS IN SWISS ALBINO MICE: LAB-BASED ACUTE TOXICITY STUDY

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**A THESIS SUBMITTED TO DEPARTMENT OF BIOMEDICAL SCIENCES, INSTITUTE OF HEALTH JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR MASTERS OF SCIENCE IN CLINICAL ANATOMY (MSc.)**

**JANUARY- 2021**

**JIMMA, ETHIOPIA**

**JIMMA UNIVERSITY**

**INSTITUTE OF HEALTH**

**FACULTY OF MEDICAL SCIENCE**

**DEPARTMENT OF BIOMEDICAL SCIENCES**

**ANATOMY COURSE TEAM**

# EFFECT OF LOCAL ARAKE ON HEPATORENAL STRUCTURES AND FUNCTIONS IN SWISS ALBINO MICE: LAB-BASED ACUTE TOXICITY STUDY

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**FEBRUARY 2021**

**JIMMA, ETHIOPIA**

## ABSTRACT

**Background:** Ethiopia is the most culturally diversified country in which a variety of traditional alcoholic beverages are consumed in different cultures and among others Arake is very popular. The effect of Arake on internal organs structures and functions is not well studied. **Objectives**: Therefore, the aim of the current study is to assess acute effect of local Arake on hepatorenal structures and functions in Swiss albino mice.

**Methods**: Twenty eight newly breaded Swiss albino mice were randomly divided into four groups. Arake was provided for the experimental groups and distilled water for control for daily for six weeks. Throughout the treatment period the mice in both groups were observed for any behavioral change and body weight was recorded on daily bases. At the end of 42 days each animal was anaesthetized with diethyl ether and blood was collected through cardiac puncture for biochemical determination such as AST, ALT, BUN and CRT as a measure of liver and kidney functions. After blood collection the mice sacrificed by cervical dislocation and abdominal cavity was opened anteriorly through midline incision of the abdomen to gain access to internal organs notably Liver and Kidneys. Wet organ weight of each organ was recorded, accessioned and immersion fixed in 10% neutral buffered formalin for histopathological investigation. The quantitative data including body weight, organ weight and serum levels of AST, ALT, BUN and CRT were analyzed using SPSS version 26 and the result presented by descriptive statistics as mean ± SEM while the difference between groups were compared using one way ANOVA Post Hoc Tukey and p-value < 0.05 was considered as a significant. The qualitative data including histopathological alterations was investigated through preparing microscopic slides which were examined under light microscope by Anatomist and Pathologist (both blinded to dose and groups).

**Results:** Twenty eight Swiss albino mice (14 Males and 14 Females) were used to start the experimentation. But, four mice (2 Male and 2 Female mice) were dead within 72hr of acclimatization and twenty four mice survived up to end of necropsy. The body weight of the treated groups was significantly decreased as compared to control group ($p<0.05$) but the decrement of weight between the treated groups was not significant. The serum level of AST

and ALT of the experimental group was significantly higher when compared with control group ($p<0.05$). Serum level of BUN and CRT was increased as the dose of Arake administered increases but the serum level of the CRT has no significance difference in all the groups but serum urea was significantly different between the groups. Arake consumption induced hepatorenal inflammation and necrosis precipitated by increment in dose accompanied by alteration of its functions.

**Conclusions:** The result of this study revealed that Arake intake decreased body weight of the mice and induced hepatorenal inflammation and necrosis accompanied by alteration of its functions. The damaging effect was exacerbated as the dose of Arake increased.

**Keywords**: Arake, Kidneys, Liver, Histopathologic alteration, Kidney Function Test, Liver Function test.

## ACKNOWLEDGMENTS

First of all, I would like to thank God of Almighty to save all of us up to now especially during this COVID-19 pandemic. I would like to acknowledge Jimma University, Institute of Health department of biomedical sciences for giving me the chance to prepare my thesis on this topic and sponsoring. Next, I would like to express my heartfelt acknowledgments to my advisor Mr. Tilahun Alemayehu (MSc, Assistant Professor of Human Anatomy) for taking his precious time by provision of valuable advice, constructive comments, and ideas starting from the beginning of this work and for his cooperativeness, unreserved willingness to share valuable scientific comments and guidance. I am also happy to extend my gratitude to Mr. Niguse Hamba (MSc in Human Anatomy) for his tangible comments and encouragement throughout this paper. I would like to express my heartfelt thanks to Mr. Bereket (PhD fellow) for helping me to specify the type of alcohol found in Arake using UV-spectrometer at Jimma University, Organic chemistry laboratory. I extend my thanks to Biochemical Engineering staff members of JIT for supporting me in alcohol concentration determination using Alcoholometer and $P^H$ using $P^H$ Meter at Biochemical engineering laboratory. In advance I want to say thanks to Dr. Dechasa Tegegne (DVM, MSc in Animal Biotechnology) for his cooperativeness and willingness to provide me constructive comments throughout this work. I am also happy to thanks to Mr. Madi Abafogi for his contribution in this work by breading the mice and rear them up to 8-10weeks. Again I want to express my heartfelt thanks to Mrs. Genet Megersa for her technical support at JUCAVM, Animal experimentation and Biotechnology laboratory during the experimentation. I would like to say thanks to Dr. Gebi Namo (MD, Pathologist) for willingness to support me by taking his precious time to examine the pathological features of the slide prepared through histotechniques. Last but not least I would like to extend my heartfelt thanks to all my instructors of anatomy course unit and friends for your valuable contribution in this work.

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| | |
|---|---|
| ADH | Alcohol Dehydrogenase Enzyme |
| AKI | Acute Kidney injury |
| ALT | Alanine Aminotransferase |
| ALD | Alcoholic Liver Disease |
| AST | Aspartate aminotransferase |
| AFLD | Alcohol-related Fatty Liver Diseases |
| ANOVA | Analysis of Variance |
| APC | Alcohol per Capital consumption |
| AST | Aspartate Aminotransferase |
| BUN | Bilirubin, Urea and Nitrogen |
| BW | Body Weight |
| CKD | Chronic Kidney Disease |
| CLD | Chronic liver Disease |
| CYP 2E1 | Cytochrome P450 2E1 |
| CRT | Creatinine |
| DALYs | Disability Activity living Years |
| FMoH | Federal Ministry of Heath |
| HBV | Hepatitis B Virus |
| HCV | Hepatitis C Virus |
| H & E | Hematoxylin and Eosin |
| $H_2O$ | Water Molecule |
| IRB | Institutional Review Board |
| JU | Jimma University |
| JUCAVM | Jimma University College of Agriculture and Veterinary Medicine |
| L | Liters |
| LVT | Liver Function Test |
| $NADH^+$ | Nicotinamide Adenine Dinucleotide |
| NCDs | Non-Communicable Diseases |

| | |
|---|---|
| OECD | Organization for Economic Cooperation and Development |
| PLC | Private Limited Company |
| PPE | Personal protective Equipment |
| RAAS | Renin-Angiotensin-Aldosterone System |
| RFT | Renal Function Test |
| ROS | Reactive Oxygen Species |
| SPSS | Statistical Package for Social Science |
| UDAW | Universal Declaration of Animal Welfare |
| WHO | World Health Organization |

# 1. INTRODUCTION

## 1.1 Background

Ethiopia is culturally diversified country in which a variety of traditional alcoholic beverages are consumed in different cultures. These homemade alcoholic beverages are traditionally prepared for local consumption by the communities(1). Among others, 'Tella', 'Qeribo', 'Geso', 'Borde', 'Tej', and 'Arake' are very common drinks. Throughout the country, 'Arake' is popular traditional drink in most cultures and consumed by most community members. In different regions and cultures, it is known with different vernacular names such as 'Araki' in Amhara, 'Qulamme' in Oromo Illubabor zone 'Qub-lammee' South West Shawa and 'Dibicho'in Sidama. Arake is a colorless conventionally produced alcohol form mass of more concentrated fermentation and commonly used alcoholic drinks by the lower social classes or lower-income particularly in the rural and semi-urban setting of the country(2).

Arake is produced at home from germinated Wheat or Barely, Bread from cereals, water and others through conventional distillation process by using a lot of firewood. Arake is a liquid "prepared from grounded Gesho leave and germinated barley or wheat powder mixed with water 2-3 days before bread (Kita) preparation; locally the mixture is called Xinsisa. Then Kita prepared from Maize or other available cereals added and fermented for 5-7 days locally called as Difdifa to be distilled into Arake. The distillation process is carried out by using firewood (traditional means) and on average each distillation times take around 40 minutes particularly in South West Shawa, Oromia regional state of Ethiopia." Key informant on June 20/2020 at 10:00 AM phone interview.

The alcoholic content of Arake is believed to be ranges between 30 and 40 % (v/v of ethanol). The alcoholic contents of Ethiopian traditional beverages "Arake" are different and this variation is based on raw materials used for spontaneous fermentation and method of producing; while the PH from 3.5-7.5 but mostly around 4(2).

Fermented beverages hold a long history in mankind and used as nutrition in many society and cultures across the world(4). Most traditional alcoholic beverages are believed as a stimulant and facilitate interpersonal relationship as a result it is commonly consumed during holidays, recreation, church festivals, funerals, invitations of guests, friends, and relatives(5).

Arake is a commercially available traditionally prepared alcoholic beverage which can pose health threats due to its high alcoholic strength and undesirable additives by resellers(6). Traditionally Arake classified into two: Tera Arake and Dagim Arake(7). Alcoholic beverages are a part of the human dietary culture and have an inseparable relationship in mankind's history and it is a way of enhancing the nutritional significance and social relationship of human beings(8).

In 2016, an estimated 2.3 billion global populations consume alcohol, resulting in 3 million death and 132.6 million DALYs(Daily Adjusted Living Years)(9). Men consume four-fold of women 18.3L of pure alcohol when compared with Women consume 4.7 L(10). Globally in 2015, unrecorded APC (Alcohol Per Capital Consumption) accounts one quarter 25.5% of total APC 1.6 L pure alcohol)(11). Different countries are moving towards controlling the consumption of alcohol globally, particularly, Ethiopia is one of the sub- Sahara African countries that have planned to reduce alcohol consumption by adding excise tax on industrially prepared beverages. The government of Ethiopia initiated an alcohol regulation program in line with African leaders' commitment to achieve prevention and control harmful alcohol use by 2030(12). Consumption of alcohol is a high risk of disease and injury and the level of alcohol consumption in the country is APC by the Adults which includes recorded and unrecorded consumption(11).

Trends in alcohol consumption are moving in an unfavorable direction particularly among women, minorities, and rural residents(13). Liver and kidney are important organs of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, and are especially vulnerable to damage(14).

The Liver is the most vulnerable organ to different insults such as metabolic, toxic, microbial, circulatory, and neoplastic(15).

The kidney is one of the vital organs which regulate water intake and outtake, balance minerals and electrolytes, and produce hormones(16). Alcohol is one of the factors that compromise

kidney function through the acute or chronic intake or as a complication of liver disease(17). Alcohol consumption is highly associated with an increase in the incidence of newly diagnosed Chronic Kidney disease (CKD) nearly by two-folds or more particularly at a young age(18).Excessive alcohol consumption is becoming a global problem and people have recognized that there is a clear relationship between CKD and alcohol consumption(19,20). As the world moves towards alcohol control, the focus of interventions has to shift to include traditional alcoholic beverages. Routine integration of traditional alcohols in alcohol control policy as part of strategies has potential outcome to make a significant contribution in the fight against alcohol-related health problems. The possibility of continuous consumption of traditional beverages is undetected due to unrecorded production or APC which shows an insignificant understanding the effect traditional alcohols. Therefore, this study helps to determine the effect of traditionally prepared alcohol “Arake” on the hepatorenal structures and functions in Swiss albino mice.

## 1.2 Statement of the problem

Alcohol intake is one of the world's leading risk factors for mortality and morbidity(19). Alcohol affects almost all the body's organs by various pathways which may be noticeable in short or long term(22). About 24.3 % of the alcohol consumed is unrecorded, untaxed and usually generated by individual families by conventional methods, according to WHO 2014 report(23). Traditional alcohols are commonly consumed on daily basis to occasion basis by the adult population which leads to a significant alteration of structures and functions in almost all organs(24). In 2016**,** alcohol is consumed by around 2.4 billion people (32.5% of the global population) or (1.5 billion men and 0.9 billion women) out of this 3 million (5.3% of all death) alcohol-attributable deaths and 131.4 million DALYs (5% of all DALYs or Disability Adjusted Living Years) occurred(25). According to 2015 WHO survey 6.13 L of pure alcohol consumed by every person at the age of about 15 years; a significant proportion of this consumption is either 28.6% or 1.76 liters/person is homemade(26). Alcohol use causes 1.7million NCD deaths, with 1.2 million death from Digestive disease and Alcoholic liver cirrhosis being more prevalent digestive diseases in 2016, attributing 607,000 deaths and 22.2 million DALYs(9).

Alcoholic liver cirrhosis is a leading causes of morbidity and mortality(27). In 2010, 493,300 deaths(156,900 women and 336,000 men) and 14,554,000 DALYs (4,112,000 Women and 10,432,000 Men) were due to alcoholic liver cirrhosis; 80,600(14,800 Women and 65,900 Men) and 2,142,000 DALYs(335,000 Women and 1,807,000 Men) were due to alcoholic liver cancer(28). More than 1.32 million deaths were caused by cirrhosis in 2017(2.4 percent of all deaths) worldwide(29). The risk of disease, injury and death associated with excessive alcohol use remains high for most countries(30). In recent years chronic disorders are specifically related to alcohol consumption in which is a modifiable risk factors(31).

Increasing alcohol consumption burden in Africa the region contributing to mortality and morbidity(32). The consumption alcohol consumption and the burden of

diseases are increasing because policymakers and population receive little attention, especially with regard to conventional alcoholic beverages(33). Growing alcohol consumption and alcohol related mortality were 2.1 % in 2000,2.2% in 2002, 2.4% in 2004(34).Total alcohol consumption were responsible for 6.4% of all deaths, and 4.7% of all DALYs(35). A study conducted in Nigeria on semi-urban dwellers community showed alcohol consumption is significantly

associated with being male, low educational status, low socioeconomic status, and unemployed and being Christian in religion(36). The great variation in strengthening alcohol control policies in African countries vary and makes the negative correlation between alcohol policies and consumption is visible(37). A study conducted in Southwest Nigeria on 145 patients on contribution of alcohol consumption to Chronic liver diseases (CLD) accounts for 4% and no-alcoholic fatty liver disease measures around 1.28% of the CLD in 2010(38).

National Survey conducted in Ethiopia on 9,800 participants in 2017 showed that the overall prevalence of alcohol consumption is 49.3% while 40.7% current drinkers and 12.4% heavy episodic drinking(30). The average APC among individual ≥15years of age in Ethiopia is 11.58% (Men) and 1.21% (women) (39). The prevalence of alcohol consumption in Ethiopia ranges from 1% (Somali) to 76% (Amhara) and the proportion of male 1% in Somali and 91% in Tigrai(40). Alcohol consumption levels and patterns from 1961-2016 showed that Beer (55%), others (37%), Spirits (8%), and less than 1% wine(41). Having financial ability to buy industrially prepared alcoholic beverages or relatively richer individuals tends to use industrially prepared alcoholic beverages but the poorest drinkers use traditional alcoholic beverages like Arake(42). In 2015 surveillance conducted in Ethiopia showed that the overall lifetime of alcohol consumption was 49.3% and 40.3% are current drinkers while heavy episodic drinkers responsible for 20.5% Male and 2.7% Female in addition male sex, rural residence, married and tobacco smoking is factors associated with alcohol consumption(30).

A study conducted in the rural district of Ethiopia on alcohol consumption showed that the overall prevalence of alcohol use was found to be 21%; 31% male and 10.4% female(43). A cross-sectional study conducted in Harar on 150 patients with CLD between April 2015-2016 on unexplained chronic liver diseases showed that 55.5% etiologic factors for CLD were not identified and alcohol abuse responsible for 2%.Unmatched Case-Control study conducted in Addis Ababa, Ethiopia on 1610 patients (812= Case with CLD and 798 without liver diseases) the etiology of the Chronic liver diseases with also associated alcohol consumption next to hepatitis virus(44).Moreover, Arake is mostly consumed by the lower socioeconomic class who cannot afford the cost of industrially prepared alcoholic drinks(45) and lose governmental attention or neglected in alcohol control policy. This leads to a lack of information in Ethiopia regarding the health effect of traditional alcoholic beverages. Ethiopian government launched an

alcohol control policy by adding excise tax to industrially prepared alcohol in 2019 and most alcohol consumers shift to use traditional alcoholic beverages particularly Arake due to cheap cost and easy of accessibility. To support the SDG of 2030 in reducing the harmful use of alcohol this study aims to examine the effect of traditionally prepared alcohol “Arake” on hepatorenal structures and functions in Swiss albino mice.

### 1.3 Significance of the study

Among many factors affecting health, alcohol consumption can be mentioned. Arake is widely consumed traditional alcoholic beverages across our country and it is thought to cause adverse health events especially when consumed repeatedly. This study attempted to identify the Acute effect Arake on hepatorenal structures and functions. So, this study delivers a right insight about the effect of Arake on health. As a result, the finding of this study may help us input information to propose appropriate adverse event of Arake. Moreover, the community can use the findings of the study to adjust its alcohol consumption pattern particularly Arake with unknown alcoholic concentration. It may also support health policy makers to include traditional alcoholic beverages (i.e. Arake) in alcohol control policy.

# 2. LITERATURE REVIEW

## 2.1 Effect of alcohol on the body weight

Alcohol consumption is a risk factor for weight gain at regular or higher (30g/d) levels of drinking but light to moderate intake has protective against weight gain in older age not in younger individuals(46). Study conducted in Croatia on the effect of wine on the weight of rat showed that the rate and extent of weight gain is higher in younger rats when compared with older(47). Study conducted in Poland in 2019 on effect of alcohol consumption on body mass gain of Wistar rat figured that regardless of acute or chronic intoxication may causes liver damage and impair amino acid uptake, protein synthesis and transport from liver which influence body weight(48). Study conducted in USA in April 2009 showed that ethanol administration for a mice can leads to reduced body weight gain(49).

## 2.2 Effect of traditional beverage on hepatic structures and functions

The liver is the largest internal organ primary responsible for alcohol metabolism which attribute the organ to alcohol related injury(50). Study conducted in Poland by Broska et al in 2003 on hepatic and kidney function in rats exposed to Ethanol showed that liver weight was slightly decreased when compared to distilled water administered rats(51). Small amount of alcohol is metabolized in gastric mucosa which contain Alcohol Dehydrogenase (ADH) gastric first path metabolism(52). Alcohol consumption is one of the predisposing factors to liver diseases starting from basic steatosis to cirrhosis including Alcoholic Fatty Liver Diseases (AFLD) and alcoholic hepatitis (42,57). Most Liver diseases in Africa are related to HBV, Alcohol misuse, HCV respectively and traditional herbal medicine causes liver fibrosis(54). Clinical alcohol related liver damage includes reversible fatty liver (fat deposition in the liver), alcoholic hepatitis or wide spread inflammation and tissue destruction or necrosis which leads to fibrosis occur in 50% of heavy drinkers; alcoholic liver cirrhosis extensive fibrosis in which and stiffness of the blood vessels and distorts the internal structures(53,55).Liver cirrhosis is a serious and characterized by diffuse fibrosis of the liver connective tissue, which causes deterioration of its structure, degeneration and destruction of hepatocytes(56). Study conducted in 2015 by Snur MA Hassan et al in Iraq on Ethanol induced hepatic and Renal histopathological change showed that mild to severe lesion(infiltration of neutrophils, pinkish exudate and vascular congestion) in both tissue section while examined under bright-field microscope and the severity of lesion is proportional

to the dose of ethanol administered(57). This structural changes are mediated by release of cytokine and inflammatory mediators, macrophage and lymphocyte activation(13,58). Fatty liver(20% of alcoholics) characterized by liver enlargement(hepatomegaly)(58).Alcohol consumption is associated with chronic liver disease through elevating liver enzymes(59). Alcohol consumption also induces alteration in liver function through moderate increment in amino transaminase which is a specific marker of alcoholic liver damage and increased in its activity persist in the serum(38,56). Hepatocyte secret and liver parenchymal cells are involved in alcohol metabolism which exposes the liver to structural change as role mediators and generation of multiple ROS(52). Kupffer contains CYP 2E1 activate gut bacteria facilitate endotoxins and lipopolysaccharides taken by the liver while hepatic stellate cells as key cells responsible for collagen synthesis in alcoholic fibrosis and cirrhosis(52,60). Study conducted in China in 2017 on alcohol induced liver injury in Mice showed that there is inflammation and tissue necrosis(61). The serum level of AST to ALT ratio tends to be the a useful index to differentiate the alcoholic liver disease from non-alcoholic steatohepatitis(62). Acute ethanol consumption increases the production of ROS and lowers the cellular antioxidant level which leads to oxidative stress or liver injury(63). Consumption of alcohol give rise to liver damage manifested acutely by elevations of serum AST and ALT level and histological alteration while prolonged consumption leads to permanent damage(64). In most liver disease the serum level of ALT is more elevated when compared with AST but in alcohol induced liver disease AST is more elevated(65,66).

### 2.3 Effect of traditional beverage on renal structures and functions

The kidney is one of the vital organs which regulate fluid intake and outtake, balance minerals and electrolytes, and produce hormones(67). Alcoholism is one of the contributing factors to kidney disease in which the normal function of the kidney is seriously altered(20). Alcohol use is highly associated with an increase in the incidence of newly diagnosed Chronic Kidney disease nearly by two folds and more particularly at a young age(18).Alcohol misuse and kidney injury ROS is one of the byproduct of alcohol metabolism can causes cellular damage (e.g. renal parenchyma) unless cleared by the antioxidants(68). Exposure of kidney to ROS is due to high polyunsaturated fatty acid in the composition of renal lipids and it leads to glomerulosclerosis and tubule-interstitial fibrosis(69,70). Ingestion of alcohol associated with various deleterious

effects on the kidney ranging from tubular dysfunction to different types of acute renal failure by reducing renal function(69,71). Alcohol consumption also alters the renal microarchitecture which includes: atrophic renal corpuscles, dilatation and congestion of the peritubular vessels, and renal corpuscles with obscure Bowman's space and a few foamy-appearing tubules(71). Acute alcohol consumption also causes diuresis of the water due to inhibition of AVP or suppression of ADH as a temporary effect(72) Ethanol itself markedly induces the expression of the microsomal ethanol oxidation system (CYP2E1), producing reactive oxygen species as a byproduct(68). Increased gastrointestinal permeability and endotoxin load may lead to alcoholic steatohepatitis resulting in excessive immunoglobulin A (IgA) load due to increased intestinal production and decreased hepatic IgA clearance accumulated in the kidney leading to glomerulopathy(73,74). Renal microcirculatory alterations in advanced liver cirrhosis lead to hepatorenal syndrome(75). Hepatorenal syndrome is alteration of kidney function due to reduced circulation, $Na^+$ and $H_2O$ retention (azotemia) which leads to spontaneous bacterial peritonitis as significant complications of cirrhosis with ascites(56). Alcohol-induced skeletal muscle damage leads to excessive amounts of circulating myoglobin, causing renal tubular injury as a result of increased oxidative stress(76). Chronic renal hypoxia develops, activating the renin–angiotensin–aldosterone system (RAAS), which in turn leads to further free radical production and to the propagation of fibrotic pathways(77). The plasma level of urea and creatinine in alcoholics slightly elevated but is not a marker of kidney injury but may occur at advanced chronic alcoholic liver disease or hepatorenal syndrome(78).

Hepatorenal syndrome is alcohol-induced in which alcoholic liver cirrhosis leads to decreased urine outflow, decreased sodium excretion, and leads to ascites(79). Excessive alcohol consumption harms the renal function in maintaining the body fluid, electrolyte disturbance, and acid-base balance. Alcohol is a diuretic or makes the kidney produce too much urine in the time of excess consumption and may leads to dehydration(80). However, the kidney expresses cytochrome P450 E1 which catabolizes ethanol with the free radical formation and can be damaged by catabolism of ethanol(81). Metabolism of alcohol is taking place in the liver and byproduct of the metabolism is excreted through the urine due to this chronic ethanol administration leads to reduced renal tubules reabsorption and decreased renal function may be associated with the ethanol-induced change in membrane composition and lipid peroxidation(17).Alcohol consumption directly affect the kidney by altering the basement of the

glomerulus and become abnormally thickened characterized by cellular proliferation; it can also leads to kidney swelling and reduced renal function and increased amount of protein, fat and water excretion(79). After reviewing existing body of knowledge, a conceptual framework shown in Fig 1 was developed.

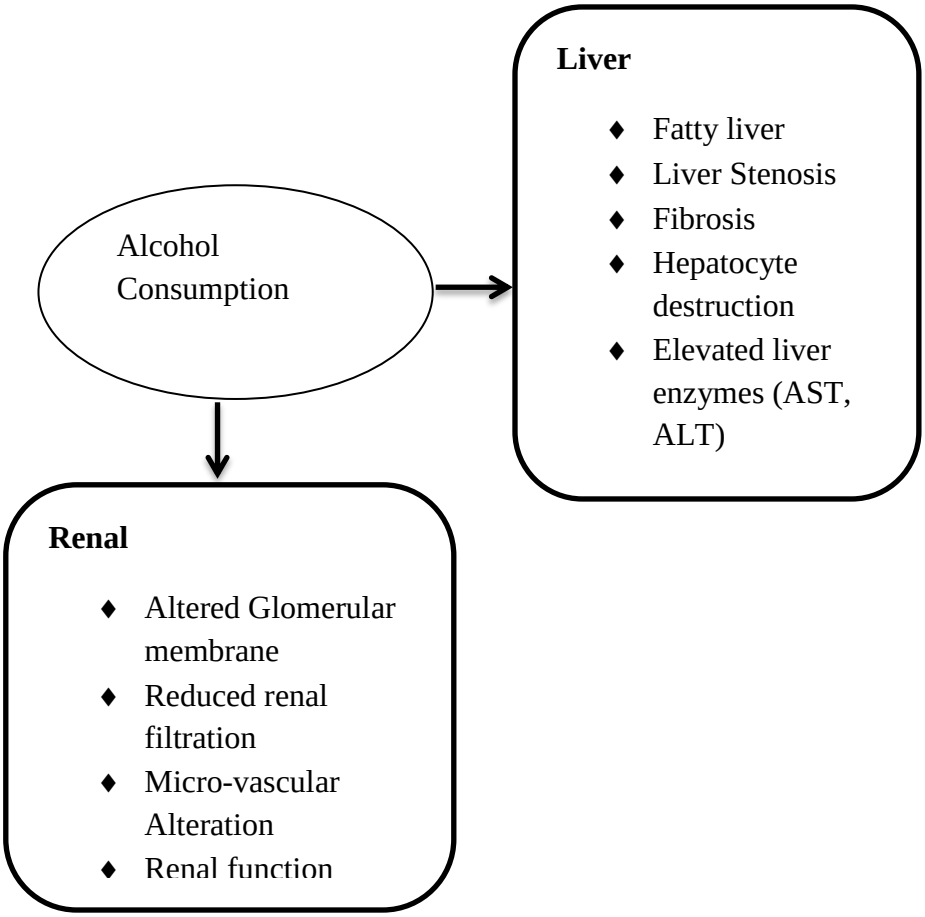


Fig1: shows conceptual frame work developed after reviewing of different literatures on changes in hepatorenal structures and functions in alcohol consume human or study animals.

## 2.4 Hypothesis

### 2.4.1 Null hypothesis

- ✓ There is no difference between experimental and control groups.

### 2.4.2 Alternative Hypothesis

- ✓ There is difference between control and experimental groups.

# 3. OBJECTIVES

## 3.1 General objective

- To assess acute effect of local Arake on the Hepatorenal structures and functions in Swiss albino mice.

## 3.2 Specific objectives

- To describe the acute effect of local Arake on the body weight of the mice
- To Investigate the acute effect of local Arake on the gross structure of the liver and kidneys
- To evaluate the acute effect of local Arake on the liver and kidneys functions
- To determine the acute effect of local Arake on the liver and kidneys histology

# 4. METHODS AND MATERIALS

## 4.1 Experimental Animals preparations and handling

Newly breeded adult Swiss albino mice were used in this study. They were obtained from and reared at Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) animal experimentation and biotechnology laboratory. Twenty- eight mice (14 male and 14 female) aged 8-10 weeks and weight of 30-42 gram was used for the study purpose. The male and female mice were housed separately in stainless-steel, aluminum cages bedded with a clean husk. The cage was cleaned with hot water and detergent twice per week to decontaminate from any microbes and husk was changed daily according to standard principles for experimental animal welfare. The temperature was maintained at 20-26$^{o}$C and a 12 hour light/12hour dark cycle. The animals were provided with a standard free mice diet (Rodent no soya) ad libitum brought from Alema Koudijs Feed PLC found in Bishoftu, Ethiopia, and clean tap water (SS sipper 250ml plastic water bottle) except when starvation is needed. Food was changed every 12hr for each group by discarding the residual rodent chow left on the feeding cabinet. Before experimentation, the mice were acclimatized to laboratory conditions for 10 days in order to minimize any non-specific stress as suggested by different scholars in various similar studies(81–83). Weight of each mouse in the all groups was measured daily basis at 0.01 precision before and after decimal place during the study period using electronic beam balance (Kern and Sohn GmbH, D-72336, Balingen Germany s.no 11316078).

## 4.2 Arake Collection and concentration determination

Arake was directly collected from the randomly selected producers to avoid manipulation by resellers found in Bacho woreda Tulu Bolo town of South West Shoa zone located 80km far from Addis Ababa in June 2020. Then its alcoholic contents using an alcoholmeter (Glass electrode with analytical sensor, USA) and $P^{H}$ were determined by using $P^{H}$ meter (Orion Model SA 72, USA) at Chemical Engineering laboratory of JIT. A calibration routine with pH 4, 7 and 10 buffers was performed prior to using pH meter then before and after using each buffer pH measurement was flushed via abundant distilled water and all tests was conducted at room temperature (85). The concentration of the Arake determined at JIT was 40% (v/v) and $P^{H}$ was found to be 3.8. The type alcohol was determined using UV-spectrometer at Post graduate Organic Chemistry laboratory of Jimma University and it has ethanol.

## 4.3 Study Design

Laboratory-based experimental toxicity study (posttest only control group design) was employed for six weeks at JUCAVM animal experimentation and biotechnology laboratory.

**Experimental Protocol**

**Group I**: 1ml/Kg distilled water

**Group II**: 2ml/Kg/BW of Arake,

**Group III**: 4ml/Kg/BW of Arake,

**Group IV**: 6ml/Kg/BW daily for six weeks. The dose or volume administered for the experimental animal 2ml/Kg/BW, 4ml/Kg/BW and 6ml/Kg/BW of 40% ethanol for the I,II and III groups respectively according to Hassan M et al, 2016 daily during the study period(83)

## 4.4 Sample Size determination and random allocation of mice

To calculate the adequate sample size required for the study Resource equation method or Crude method were used. This method is based on ANOVA in which Df(E) (Degree of freedom) lies between 10 and 20 which mean adequate to use number of animals between 10-20(83).

E = Total no of animals – Total number of groups E = (Total no of animals in each group X Total number of groups) – Total number of group E = (6X 4) – 4 => 20 which mean it is more adequate to conduct the study on 24 animals. Therefore the sample size for this study was 24 Swiss albino mice.

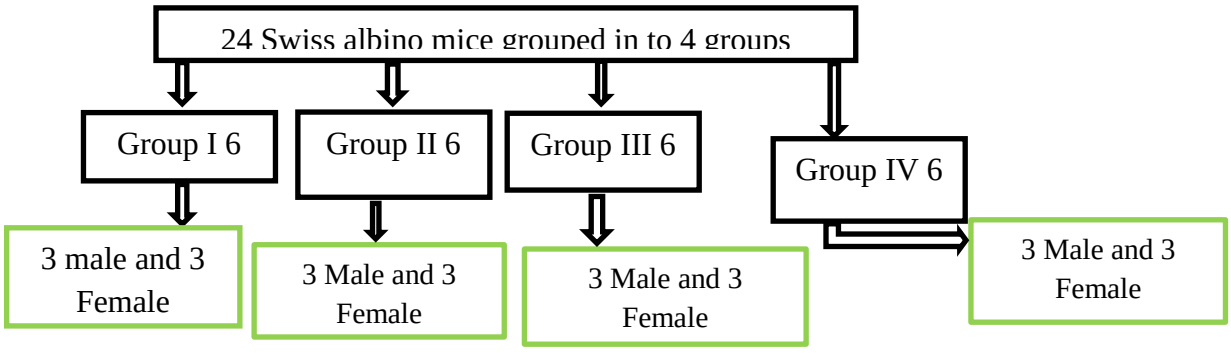


Figure 2: Random allocation of mice in to groups and experimental flow chart showing dose/kg/bw

## 4.5 Study Variables

### 4.5.1 Dependent Variables

- Hepatorenal gross morphology (color and size)
- Body weight
- Hepatorenal histopathologic change
- Renal function test (BUN and Creatinine)
- Liver function test (AST and ALT)

### 4.5.2 Independent Variable

- Dose of Arake in ml/Kg

## 4.6 Blood and organ sample collections

After six weeks of treatment, overnight starvation was carried out and each mouse was anesthetized with diethyl ether inhalational anesthesia in a glass container. Then 1.5-2ml of blood sample was collected through cardiac puncture using 25G, 3ml disposable syringe with needle through trans-abdominally. The collected blood sample were transferred to in a yellow jelly test tube with coagulating jelly to investigate liver function (the activities of ALT and AST in the serum) and renal function (Serum Creatinine and BUN) using automated analyzer Jaffer reaction(ABX Pentra 400, China). Then the mice sacrificed by cervical dislocation before incision to minimum pain or distress(86).

## 4.7 Serum biochemical analysis or hepatorenal functions test

Serum separation was preceded by using the following steps. First Clotting process then centrifugation (Gemmy Industrial Corp, Taiwan) process at end serum separation from the top of the test tube by using a transfer pipette. The serum biochemical markers of Liver function test include ALT and AST particularly in alcohol-induced liver injury(87). Alcohol toxicity is a diagnosis when ALT and AST are greater than three times normal in alcohol consumers(88). The reagent cuvette was incubated at a temperature of (37ºC ± 0.5 ºC) for the duration of the test, then 50µl of serum sample was mixed with 500µl of the working reagent and incubated for 1 min at 37 ºC. The absorbance was read at 340 nm exactly after 1, 2, and 3 minutes. Alanine Aminotransferase (ALT) and Aspartate aminotransferase (AST) was assayed by the IFCC method without pyridoxal phosphate using the Elitech kit. ALT and AST activity levels are

calculated in UI/L at 37°C. ALT and AST measurements were done using automated chemistry analyzer (ABX Pentra 400, China) spectrophotometer, wavelength 340 nanometres, in a quartz cuvette with 1 cm path length, for 175 seconds after 50 seconds of incubation in 37°C. The conversion of NADH to NAD+ correlates with serum ALT activity, and which is determined by continuously monitoring the loss of NADH absorbance at 340 nm by spectrophotometer(87).
The Renal function test is also a biochemical analysis for renal biomarkers such as BUN and Creatinine(89). Creatinine is the most common markers of glomerular filtration and its elevation significantly shows a reduction in glomerular filtration rate and serum urea also increased in acute kidney injury or clearance impairment(90).

### 4.8 Tissue processing for Histopathological study

Sample accessioning at the end of 42 days, the hepatic and renal tissue portion was taken via the neck to pubic incision using a sterile surgical blade then preserved in transparent glass container filled with a 10% NBF (neutral buffered formalin) solution and labeled with a specific identification (coded) for gross examination by pathologist and histopathological processing. The after fixation the tissue was washed with water to remove excess fixatives and dehydrated with ascending alcohol of 70%, 90% for 2 hours in each and absolute alcohol I, II, III for one and half hours (I, II) and overnight (III). The dehydrated tissue was cleaned with xylene in two stages for 1 and a half hours and 2 and a half hours. Then tissue was infiltrated with three changes of paraffin wax-for one and a half hours, two and a half hours, and overnight.

Finally, the tissues were inserted into paraffin wax in square plastic plates (Cassette) forming tissue blocks, whereby each tissue block was labeled and preserved at room temperature until sectioned. The tissue blocks was cut into ribbon Microtome (Leica Model: TP 1020, Germany at the pathology lab of Jimma University) at a thickness of 5$\mu$m and the section was collected at every $5^{th}$ then put on to the surface of a warm water bath (Mounting bath Model: MH85234 China) of the temperature of 37-40◦C. The floating ribbons over the surface of warm water were placed on to pre-cleaned slides and stretched out with egg albumin. The slides containing paraffin wax (Wax dispenser Electrothermal Model: MH8523, China) was arranged with in the slide holder and placed in an oven with a temperature of 40◦C for about 10 minutes to fix the tissue to the slides and allowed to cool at room temperature for 30 minutes and stained with Hematoxylin for 5 minutes and then eosin for 15 second. Two sets of coupling jars were

prepared for regular H and E staining; 1) for paraffin removal and hydration and 2) for dehydration and clearing. In order to extract the paraffin the tissue, placed in xylene I for 5 minutes and xylene II for 2 minutes and hydrated with descending concentrations of 95% alcohol for 2 minutes in each, 70% of alcohol for 3 minutes and 50% alcohol for 5 minutes.

The tissue sections were washed with tap water for 5 minutes and stained with Hematoxylin for 6 minutes. The slides were submerged in acidic alcohol for a second for distinction and controlled over stained hematoxylin then situated in the bluing solution until they became blue. Upon the slides were counterstained with eosin for 15 seconds and then washed in tap water for two minutes. The sections were dehydrated with an increasing alcohol concentration of 50%, 70%, and 95% for two minutes.

For three minutes in each, the dehydrated parts were cleared with xylene I and xylene II, permanently placed on microscopic slides using DPX and cover slips, and then observed under the light microscope to investigate any histological change (histologist and pathologist), thus contrasting the histology of the treated groups with that of the control group. Upon examination the photomicrographs of selected slides of liver and kidney from both the treated and control mice were taken under a Light microscope (Olympus, CX21FS1, Philippines) with magnification power(40X) and the slides Photomicrographs were taken objective using an automated built-in digital photo camera (Camera KRUSS optronic Germany 3.0 MP USB 2.0 Histology lab of Jimma University).

## 4.9 Data Quality Management

Trained Laboratory technician and principal investigator were handled the animal during the study period and provide the treatment according to the protocol. The histotechniques principles (during tissue collection, preservation, processing, and slide preparation) and laboratory protocol (for liver and renal function test) were strictly followed. Experienced histologist (Anatomist) and pathologist were blinded to the groups and the dose to eliminate observer bias before examining the prepared microscopic slides under bright field microscope with different magnification power. All prepared slides were re-examined to assure the consistency of the finding. The numerical data such as body weight, relative organ weight, BUN, CRT, AST and ALT entered into SPSS version 26 was rechecked before analysis.

## 4.10 Data Analysis

All Raw data from each group was entered into Statistical Package for Social Sciences (SPSS) software version 26 for further analysis. Statistical analysis of variables such as body weight, relative organ weight, BUN, CRT, ALT, AST, etc. were presented by descriptive statistics or standard error of the mean. The differences between the experimental and control groups was compared using one-way analysis of Variance (ANOVA) with Post Hoc equal variance assumed Tukey and P-value $< 0.05$ was considered as significant and used to test the hypothesis. Microscopic evaluation were qualitative analysis carried out by Senior Pathologist an Anatomist through preparing microscopic slides for each group and presented in form of photomicrography.

## 4.11 Ethical Considerations

The letter of starting the research was taken from the Biomedical Department and ethical clearance or ethical approval from IRB (Institutional Review Board) of JU to get permission to conduct the research/ Ref.No. IHRPGD/835/2020 (ANNEX-VI). In any session of this research, Covid-19 prevention was considered by Hand washing, using PPE (Face mask) and physical distancing.

All procedures and principles were strictly reviewed by Doctor of Veterinary Medicine to ensure procedures are appropriate and humane. Adverse effect of Arake was prevented by providing after feeding and dose were adjusted by weighting each mouse before giving. Unnecessary duplication of the experiment were avoided by following the procedure or protocol well(91). The mouse were handled in a way that improving animal welfare to minimize distress and pain which includes: hunger, thirst, abnormal cold or heat, injury in any time of the study, and anesthesia was provided and checked for pain sensation by pinching tail or over skin before dissection or euthanasia. The principal investigator and animal lab investigator was wear personal protective equipment before performing any procedure on the Swiss albino mice. After completion of the experiment and procedure, any remnant body parts of the mice were discarded/buried in sealed plastic containers according to the Universal Declaration of Animal Welfare (UDAW) to prevent environmental contamination(86).

## 4.12 Dissemination Plan

The research result report will be presented to Jimma University Institute of health and graduate program coordinating office research defense and other national and international scientific conferences. Moreover, it will be also published on international reputable scientific journals.

# 5. RESULTS

Tissue and blood sample were collected from all survived animals and the main findings were presented as follow.

## 5.1 Body weight of the Mice

The paired t-test was used to compare the initial and final body weight change of the mice. The mean final body weight of Group I mice or control (34.50 ±2.35 g) was increased higher when compared with final mean body weight of group II (30.17±2.86 g) ($P<0.05$). Similarly, the final mean body weight of Group II or 2ml/Kg (30.17 ± 2.86 g) slightly increased even though less increment when compared with group I (34.5 ± 2.35g) and Group III (27.17 ± 3.60 g) mice was slightly decreased but it was not significant ($P>0.05$). Furthermore, the final mean body weight of Group IV mice or 6ml/Kg (28.00 ± 2.00 g) was decreased when compared with their initial body mean body weight (29.67±1.63 g) ($P< 0.05$).

Table 1: Effect of acute oral administration of Arake on mean body weight of the mice

| Groups | Number of mice | Initial Mean BW(g) | Final Mean BW (g) | Mean difference ±SD | P- Value |
|---|---|---|---|---|---|
| I | 6 | 28.50 ± 2.88 | 34.5 ± 2.35 | 4.17 ± 0.93 | 0.004 |
| II | 6 | 27.83 ± 2.48 | 30.17 ± 2.86 | 1.81±1.91 | 0.068 |
| III | 6 | 29.00 ± 4.15 | 27.17 ± 3.60 | 0.52±1.26 | 0.363 |
| IV | 6 | 29.67 ± 1.63 | 28.00 ± 2.00 | 1.45±2.19 | 0.001 |

The results were expressed in as Mean ± SD. Values with different superscripts within the same column are statistically significant at ($p< 0.05$)

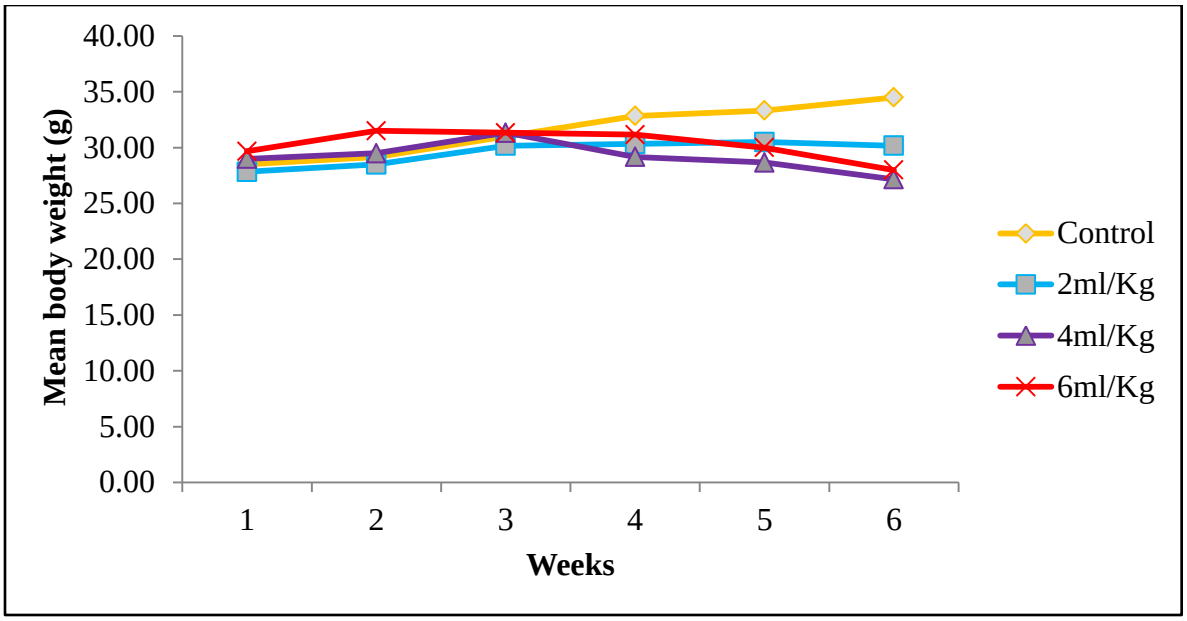


Fig 3: Effect of Orally administered Arake (2ml/Kg, 4ml/Kg and 6ml/Kg) on the body weight of the mice in weeks line graph

## 5.2 Effect of Arake on organ weight

### 5.2.1 Liver

Gross examination (color and size) of liver after removed from mice were examined by observing for color change and size were measured by senior pathologist has no gross difference when compared. All liver tissue removed from mice among all the groups are similar in color but the size is different depend on the body weight of the mice and amount of Arake administered.

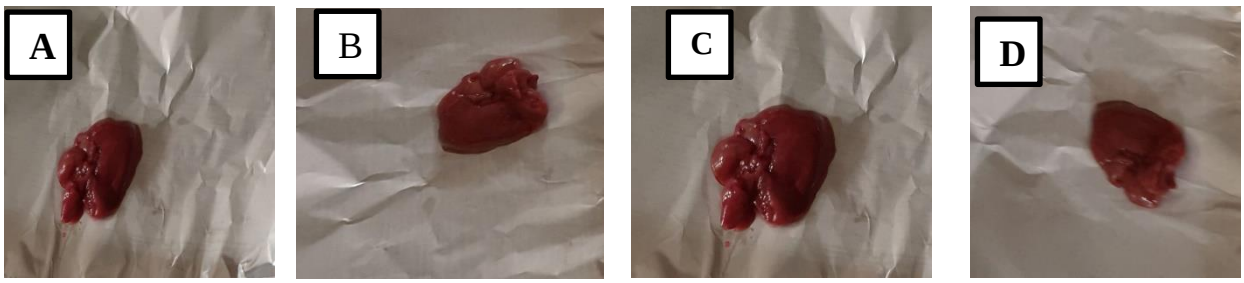


Figure 4: Gross morphology of the liver of control 1ml/Kg distilled water (A), Exp1 2ml/Kg (B), Exp2 4ml/Kg(C) and Exp3 6ml/Kg of Arake respectively.

The relative liver weight of group I, group II, group III, and group IV mice were (6.93 ±1.10 g), (7.90 ± 0.41 g), (8.74 ±0.55 g) and (9.00±0.63 g) respectively. According to this study, the relative weight of the liver was significantly different between certain groups. Group I mice

relative liver weight (7.00 ±1.10 g) was statistically different from Group III (8.74 ±0.52 g) and IV (9.00±0.63 g) but, there was no statistical significance difference when compared with Group II (7.90 ± 0.41 g) mice even though it was slightly higher. Similarly, Group II relative liver weight (7.90 ± 0.41 g) was not significantly different from Group I, III and IV even though there was increment in liver weight simultaneously with increment in dose of Arake.

Table 2: Effect of Acute oral administration of Arake on the absolute and relative weight of the liver of mice

| Groups | Absolute Liver weight (g) | Relative Liver weight (g) |
|---|---|---|
| I | $2.46 \pm 0.47^{b}$ | $6.93 \pm 1.10^{b}$ |
| II | $2.42 \pm 0.31^{b}$ | $7.90 \pm 0.40^{b}$ |
| III | $2.42 \pm 0.43^{b}$ | $8.74 \pm 0.52^{b}$ |
| IV | $2.52 \pm 0.29^{b}$ | $9.0 \pm 0.51^{b}$ |

The result expressed as mean ±SD. Values with different superscripts within the same column are statistically significant (a) or ($p < 0.05$) MD=Mean Difference, SEM= Standard Error of Mean

### 5.2.2 Kidneys

Gross examination (color and size) of Kidneys after removed from mice were examined by observing for color change and size were measured by senior pathologist has no gross difference when compared.

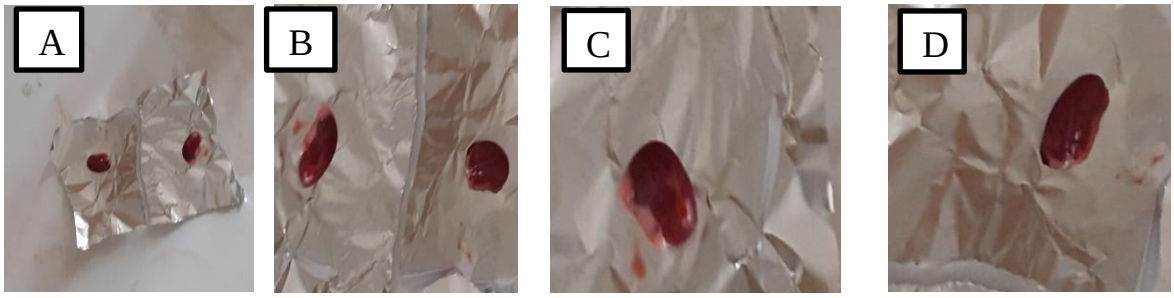


Figure 5: Gross morphology of the Kidneys of control 1ml/Kg distilled water (A), Exp1 2ml/Kg (B), Exp2 4ml/Kg(C) and Exp3 6ml/Kg of Arake respectively.

The relative weight of both right and left kidney has no significant difference between all the groups. Group I mice relative liver weight for the right and left kidney respectively (1.33±0.82 g, 1.00±00g) was statistically has no significant difference from Group III (1.00±00g, 1.00±00 g) and IV (1.08±0.41g, 0.96±0.20) and also when compared with Group II (1.00 ± 00g, 1.00±00g) mice, it was slightly higher. Similarly, Group II relative kidney weight (1.00 ± 00g, 1.0000g)

was not showed significantly different from Group I, III and IV or with increment in dose of Arake.

Table 3: Effect of acute oral administration of Arake on the relative Kidney weight of mice

| Groups | Absolute weight of Rt Kidney(g) | Relative Rt Kidney Weight(g) | Absolute weight of Lt Kidney(g) | Relative Lt Kidney weight (g) |
|---|---|---|---|---|
| I | $0.30 \pm 0.29^{b}$ | $1.33 \pm 0.82^{b}$ | $0.21 \pm 0.12^{b}$ | $0.83 \pm 0.41^{b}$ |
| II | $0.26 \pm 0.61^{b}$ | $1.00 \pm 00^{b}$ | $0.26 \pm 0.61^{b}$ | $1.00 \pm 00^{b}$ |
| III | $0.25 \pm 0.10^{b}$ | $1.00 \pm 00^{b}$ | $0.24 \pm 0.74^{b}$ | $1.0 \pm 00^{b}$ |
| IV | $0.24 \pm 0.15^{b}$ | $1.08 \pm 0.41^{b}$ | $0.25 \pm 0.10^{b}$ | $0.96 \pm 0.20^{b}$ |

The result of absolute and relative organ weigh expressed as mean ±SD (g). Values with different superscripts within the same column are statistically significant ($p < 0.05$)

### 5.3 Liver Function biomarkers

According to the study there was a significance difference of serum AST and ALT levels between control and experimental groups. There was a significant increment of serum ALT and AST of the experimental groups when compared with control group.

The serum AST level of Group-III mice (169.83±15.91 U/L) was increased significantly when compared with the serum AST level of Group-II mice (87.67±38.85 U/L) and the serum AST level of Group-IV mice (145.75±6.51 u/l) was also increased when compared with the serum AST level of Group-III mice but it was not significant ($P > 0.05$). Similarly, the serum AST level of Group-III mice (169.83±15.91U/L) was increased significantly when compared with the

serum AST level of Group-II mice (87.67±38.85 U/L) ($P<0.05$) and the serum AST level of Group-IV mice (178.50±12.42 U/L) was also increased when compared with the serum AST level of Group-III mice (178.50±12.42 U/L) but it was not significant ($P > 0.05$). Regarding the AST level of Group-II mice (87.67±38.85 U/L) was increased significantly when compared with the serum AST level of Group-I mice (43.17 ±10.60 U/L) and the serum AST level of Group-IV mice (178.50±12.42 U/L) was also increased when compared with the serum AST level of Group-III mice (169.83±15.91) but it was not significant ($P > 0.05$). The serum ALT level of Group-I I (61.33±29.87 U/L) mice was insignificantly greater than the serum ALT level of Group-I mice (19.83±11.92 U/L) ($P > 0.05$). The serum ALT level of Group-IV mice (71.00 ±13.91U/L) was when compared with the serum ALT level of Group-III (46.67 ±21.50 U/L) but, it was not significant ($P > 0.05$).

Table 4: Effect of Acute oral administration of Arake on some liver function parameters

| Groups | Biochemical analysis of liver function | |
|---|---|---|
| | AST(U/L) | ALT(U/L) |
| I | 43.17 ±10.60 | 19.83±11.92 |
| II | 87.67±38.85 | 61.33±29.87 |
| III | 169.83±15.91 | 46.67 ±21.50 |
| IV | 178.50±12.42 | 71.00 ±13.91 |

## 5.4 Kidney function biomarkers

The serum urea level of Group-III (57.00±16.95 mg/dl) and Group-IV mice (70.17±9.00 mg/dl) was increased significantly when compared with Group-I mice serum urea level (16.00 ±9.74 mg/dl) ($P < 0.05$). In the same way, the serum urea level of Group-IV (40.83±0.12 mg/dl) mice was increased significantly when compared with the serum urea level of Group-I (control group) mice (39.59±0.22 mg/dl) ($P < 0.05$). Similarly, the serum urea level of Group-II (57.00±16.95 mg/dl) and Group-III (57.00±16.95 mg/dl) mice was also increased when compared with the serum urea level of Group-I but not significant ($P > 0.05$). There was increment in the serum creatinine level of experimental groups when compared with the control group but not significant ($P > 0.05$). Among experimental groups, the serum creatinine level of Group-III (1.00± 00 mg/dl) and Group-IV (1.00± 00 mg/dl) mice was increased when compared with the serum creatinine level of Group-II mice (0.83±0.41mg/dl) ($P > 0.05$) but the difference between all groups was insignificant ($P > 0.05$).

Table 5: Effect of acute oral administration of Arake on some renal function parameters

| Groups | Biochemical analysis of kidney damage | |
|---|---|---|
| | BUN (mg/dl) | Creatinine (mg/dl) |
| I | 16.00 ±9.74 | 0.67±0.52 |
| II | 40.83±17.87 | 0.83±0.41 |
| III | 57.00±16.95 | 1.00± 00 |
| IV | 70.17±9.00 | 1.0 00 |

## 5.5 Histopathological findings

### 5.5.1 Liver

In this study, the liver tissue of the control group has a normal appearance when compared with experimental. There were inflammations and necrosis in the liver tissue of the group II, III and IV mice. In addition to inflammation and necrosis there were shrinking of hepatocytes and fibrotic tissue observed in group-III and IV mice.

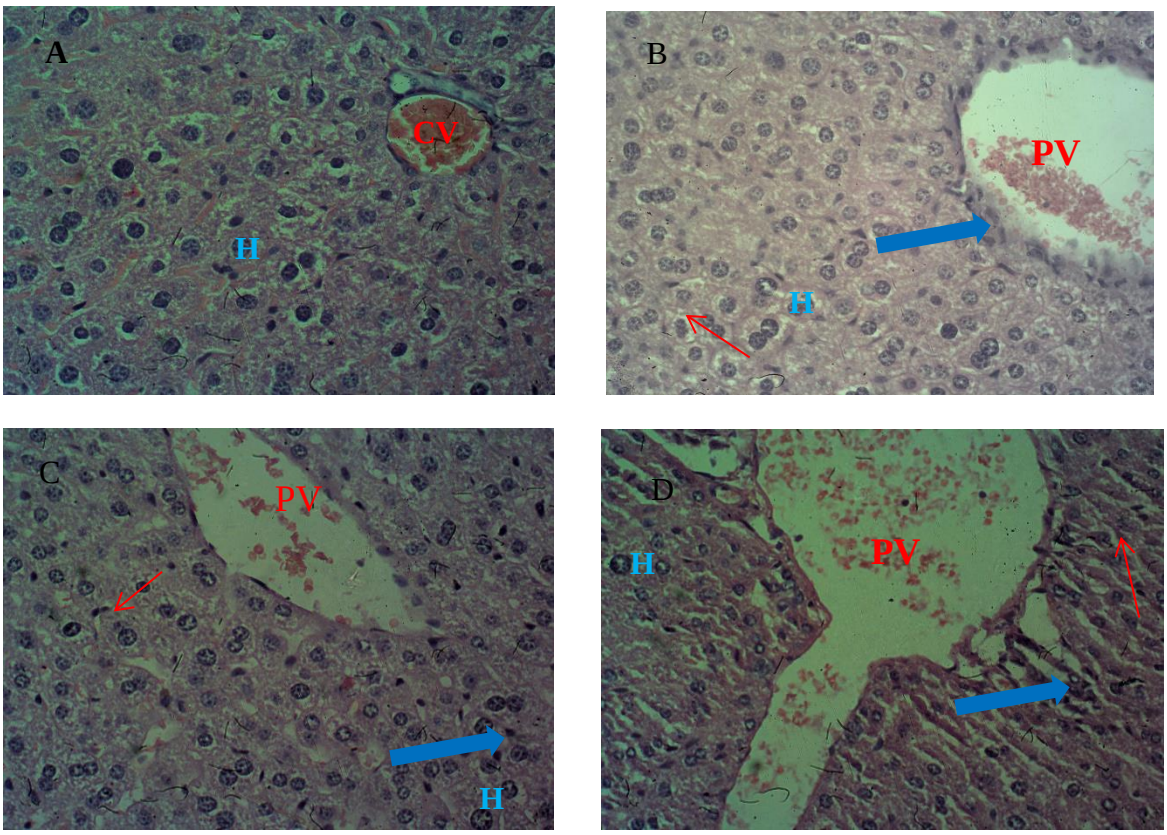


Figure 6: The photomicrographs of Liver section taken from mice and stained with H & E and all photomicrographs taken at magnification 40X. A) Control group treated with 1ml/Kg/BW distilled water shows normal liver tissues and CV: Central Vein H: hepatocytes nucleus B) 2ml/Kg/BW Arake shows mild inflammation and necrosis around the portal vein (green arrow) C) 4ml/Kg/BW of Arake moderate inflammation and necrosis (green arrow): (D) and 6ml/Kg/BW of Arake shows severe inflammation and necrosis (green arrow). The Red arrow indicates a condition of small fat droplets and Circle shows hepatocyte necrosis in alcohol treated groups. PV= portal vein

### 5.5.2 Kidneys

In this study, the kidney tissue of the control group has a normal histological appearance. But, there were histopathological changes in the kidney tissues of the experimental groups. There were inflammation, fat accumulation, obscure bowman's space, foamy appearing and necrosis of renal parenchyma in mice administered with Arake during the study period. The degree of

inflammation and necrosis is exacerbated by as the dose of Arake was increased as pathologist investigates the prepared microscopic slides under different magnification power using Microscope.

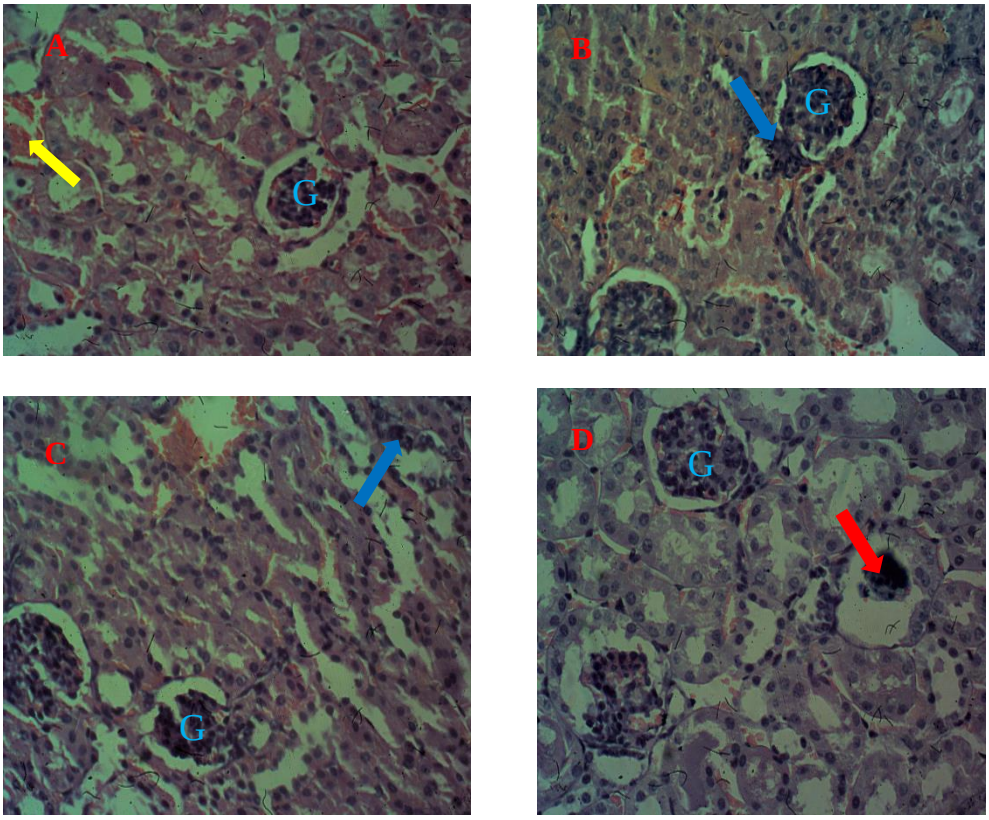


Figure 7: The photomicrographs of Kidneys section taken from mice and stained with H & E and all photomicrographs taken at magnification 40X. **A)** Control group treated with 1ml/Kg/BW distilled water shows normal renal architecture glomerulus (G) and renal blood vessels (yellow arrow) **B**) 2ml/Kg/BW treated mild inflammation and necrosis (green arrow) **C**) 4ml/Kg/BW treated shows moderate inflammatory infiltrations and necrosis (green arrow) **D**) and 6ml/Kg/BW treated shows obscured and necrotic glomerulus (red arrow).

# 6. DISCUSSIONS

## 6.1 Body weight of the mice

Several factors including: genetic, environmental, physiological and behavioral factors influence body weight(92) and inappropriate alcohol intake is also one of the factors that lead to body weigh change(92,93). The final mean body weight of 2ml/Kg treated was slightly decreased less when compared to 4m/Kg treated mice but it was not significant ($P>0.05$). Furthermore, the final mean body weight of 6ml/Kg treated mice was decreased when compared to other treatment and control groups ($P< 0.05$).

In this study, the body weight of the treated groups slightly decreased as the dose of Arake provided was increased. The findings of this study is in contrast to study done in Poland by Alexandra K et al in 2019 and his colleagues on influence of alcohol consumption on body mass gain in growing adolescent Wistar rat(48). The variation may be due to species variation between mice and Wistar rat and alcohol consumed may have physiochemical difference (ingredients). However the finding of this study is consistent with study conducted in Croatia in 2017 by Tong Zhou et al on the protective effects of lemon juice on alcohol induced liver injury revealed that ethanol consumption reduces body weight in mice(61).

## 6.2 Relative weight of the liver

The relative weight of the liver was significantly different between certain groups. 4ml/Kg treated mice absolute and relative liver weight were not significantly different from 2ml/Kg and 6ml/Kg treated groups mice even though it was slightly higher. Similarly, 2m/Kg treated absolute and relative liver weight was not significantly different from 4ml/Kg and 6ml/Kg treated groups even though was increment in liver weight simultaneously with increment in dose of Arake. This finding is in contrast with study done in Poland in 2003 by Broska and his colleagues on Rats administered with ethanol shows not weight difference. This variation may be due to difference in treatment (ethanol with cadmium) and duration of treatment(70).

## 6.3 Relative and absolute weight of the kidneys

The relative and relative weight of both right and left kidney has no significant difference between all treated groups. 4ml/Kg treated mice absolute and relative kidney weight has no

significance difference when compared with 6ml/Kg or 2ml/Kg treated groups. Similarly, 2ml/Kg treated absolute and relative kidney weight was not showed significantly difference from 4ml/Kg and 6ml/Kg treated groups. This finding is also comparable with study done in Poland in 2003 by Brzoska and his friends on effect of ethanol and on the relative kidney weight revealed that absolute and relative weight of kidney similar across treated mice when compared to this study(51).

## 6.4 Liver function parameters

The amount of AST and ALT in the serum directly related to the amount of damage to the tissue(64). The serum AST level of mice treated with 4ml/Kg (169.83±15.91 U/L) was increased significantly when compared with the serum AST level of mice treated with 2ml/Kg (87.67±38.85 U/L) but less when compared with the serum AST level of mice treated with 6ml/Kg (145.75±6.51 u/l) but it was not significant ($P > 0.05$). The serum AST level of 6ml/Kg treated mice (178.50±12.42 U/L) was higher when compared to 4ml/Kg treated (169.83±15.91) but it was not significant ($P > 0.05$). The serum ALT level of 2ml/kg treated mice (61.33±29.87 U/L) was less when compared to 6m/Kg treated mice (71.00 ±13.91U/L) and 4ml/Kg treated mice (46.67 ±21.50 U/L) even though it was not significant ($P > 0.05$). The serum level of AST increased in the experimental groups when compared with each other as the dose of Arake administered increases. The findings of this study supports previous study done by Darius S et al and his colleges in Minnesota on the ratio of AST and ALT as a potential indicator for differentiating non-alcoholic steatohepatitis from alcoholic liver diseases(60,62) and the elevation of this liver enzymes in the serum is a biochemical analysis as indicator of liver tissue damage(53,55,63,94).

As revealed from the result of this study the level of liver damage has directly related with the dose of Arake consumed by the mice. The finding of this study is similar with study done by Campollo R and Day *et al* which describes that alcohol has damage effect on the hepatic tissue(53,95,96). The damage to the liver tissue is due to the toxic substance (ROS) released in alcohol metabolism in hepatocytes which stress the hepatic tissue, fat accumulation and necrosis(28,64,97,98).

## 6.5 Kidney function parameters

As shown in the Table 6 above the serum urea level of 4ml/Kg (57.00±16.95 mg/dl) and 6ml/kg treated mice (70.17±9.00 mg/dl) was elevated. In the same way, the serum urea level of 2ml/Kg treated mice (40.83±0.12 mg/dl) was increased when compared to 4ml/Kg (57.00±16.95 mg/dl) but not significant ($P > 0.05$).

This study also revealed that there were comparable serum creatinine levels across experimental groups. For instance, 4ml/Kg (1.00± 00 mg/dl) and 6ml/Kg (1.00± 00 mg/dl) mice was the same when compared with the serum creatinine level of 2ml/Kg treated mice (0.83±0.41mg/dl) ($P < 0.05$). The findings of this study are similar with the results of previous studies done on rats which emphasize the effects of co-exposure to atrazine and ethanol on the oxidative damage of kidney(99). The damage of the kidney of the mice could be due to formation of free radicals (ROS) during alcohol metabolism in the hepatocytes which produces oxidative stress to the cell(76,78).

## 6.6 Histopathological findings

In addition inflammations and necrosis in the liver tissue of the 2ml/Kg, 4ml/Kg and 6ml/Kg treated mice; there were also shrinking of hepatocytes and fibrotic tissue observed in 4ml/Kg and 6ml/Kg treated groups. The finding of this study is consistent with study done in China in 2017 by Tong Zhou et al on alcohol induced liver injury and study done in 2015 in Iraq on Ethanol induced hepatic and renal histopathologic change showed that there were peripheral inflammation and necrosis when prepared slides investigated under bright field microscope(57,61). The reason may be due to alcohol induced proinflammatory mediators (Cytokine), circulating endotoxins and oxidative stress mediated by free radicals ($OH^-$ and $H_2O_2$) which are toxic to cells.

This study revealed that inflammation, obscured bowman's space, foamy appearing and necrosis of renal parenchyma in mice administered with 2ml/Kg, 4ml/Kg and 6ml/kg Arake during the study period as investigated by senior pathologist and the degree of inflammation and necrosis is exacerbated by as the dose of Arake was increased. This finding is similar with study done in USA on rats in 2012 by Narin F et al. and Ifudu et al in 2014 in Nigeria alcohol induces alteration in renal parenchyma by inducing inflammation and shrinking bowman's space (72,101). The reason may be due to alcohol induced oxidative stress (ROS) polyunsaturated fatty

acid in composition of renal lipids has a degenerative effect on the renal tissue by inducing inflammation and necrosis and acid-base balance abnormality(17,70).

## 7. LIMITATIONS OF THE STUDY

This study has the following limitations:

- It didn't identify the physiochemical analysis of the Arake.
- Posttest control didn't investigate pre-experimental structures and functions.
- Appetite was not tested in this study even though weight presented as a result.
- This study didn't identify the $LD_{50}$ and $LD_{100}$

# 8. CONCLUSIONS AND RECOMMENDATIONS

## 8.1Conclusions

In this study, Arake had a significant effect on the of body weight of the mice at higher dose ( 4ml/kg or 6ml/Kg) even though it slightly increase body weight at lower dose (2ml/kg) and increases the serum level of AST and ALT of liver enzymes, elevate the serum level of BUN and Creatinine in depend on the dose administered in the Swiss albino mice. In addition Arake consumption also damages the liver and kidney tissue by inducing inflammation and tissue necrosis precipitated by the dose administered followed by functional alterations. Thus, damaging effect of the Arake was approved through biochemical analysis and histopathological evaluations. This study revealed that Arake prepared at home by conventional means and consumed by the community has adverse events on hepatorenal structures and functions in Swiss albino mice.

### 8.2 Recommendations

As an investigator, I recommend the following suggestions

- Comprehensive investigations of the physiochemical of Arake should be done to identify its health risks.
- Health information should be initiated for the community to create awareness about the health risk of Arake consumption.
- Health policy makers should have to give emphasis to traditional alcoholic beverages especially Arake in alcohol control as that of industrially prepared.
- Further studies should be done to examine the effect of Arake on other organs to determine the overall health effects.

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**ANNEX-I Materials and solutions used for the study**

The following materials were used during researching at JUCAVM animal and biotechnology laboratory and histology lab of JU.

- Alcohol of different concentration
- Automated slide strainer
- Stainless plastic Cages
- Automated tissue processor
- Needle with Syringe
- Coverslip
- Hematoxylin and Eosin for staining
- Formalin 10%
- Glass slide, slide cover(cover slipe), mounting solution
- Light microscope
- Measuring cylinder
- Microscopic slide
- Oil immersion
- Blunt needle
- Paraffin Wax
- Microtome with blade to section the tissue
- Tissue processor machine
- Feeding cabinet
- Surgical glove and blade
- Tissue floating bath
- Weight scale
- Xylene

**ANNEX-II Activities recording checklist during experimental period**

**Checklist of activities on daily base from data collection to report writing period**

**Topic**: Effect of Arake on the hepatorenal structures and functions in Swiss albino mice. Laboratory based acute toxicity study design.

**Oct/2020**

***Part I***: Research setting (the laboratory was controlled according to the criteria listed on the study temperature, light/dark, well ventilation) and animal handling ethics were strictly followed at any time of the data collection.

A. Study animals were observed on daily basis for range of locomotion (exercise) and behavioral change i.e. hunter/vicious mice for the group was separated from the group and placed in separated cage. i.e. locomotion running speed and pattern, environmental factors(temperature, light/dark cycle), sex also influence exercise female run fast and longer due estrogen hormone is necessary for running behavior, climbing behavior

Table 6: Daily activity recording table during experimentation

| S.no | Locomotion and behavior | Mice in the groups | Locomotion and behavior | | Remark |
|---|---|---|---|---|---|
| | | | yes | no | |
| 1 | Exercise(mov't and running) | G1 | | | |
| 2 | Pattern and speed | G2 | | | |
| 3 | Climbing | G3 | | | |
| 4 | Hunter(behavior) | G4 | | | |

A. Weight of the each mice in the group was recorded on daily basis by using electronic beam balance (kern and sohn GmbH, D-72336, balingen Germany s.no 11316078) and recorded on the recording sheet.

Table 7: Weight recording sheet during data collection period of the study Sep 2020 Jimma, Ethiopia.

| Wgt each group(gm) | Week ► / mice ▼ | 1st | 2nd | 3rd | 4th | Total weight of the group | Mean weight of the group | Remark |
|---|---|---|---|---|---|---|---|---|
| Group 1 | M1 | | | | | | | |
| | M2 | | | | | | | |
| | M3 | | | | | | | |
| | F1 | | | | | | | |
| | F2 | | | | | | | |
| | F2 | | | | | | | |
| Group 2 | M1 | | | | | | | |
| | M2 | | | | | | | |
| | M3 | | | | | | | |
| | F1 | | | | | | | |
| | F2 | | | | | | | |
| | F3 | | | | | | | |
| Group 3 | M1 | | | | | | | |
| | M2 | | | | | | | |
| | M3 | | | | | | | |
| | F1 | | | | | | | |
| | F2 | | | | | | | |
| | F3 | | | | | | | |

***Part II***: Dependent and independent variables was determined prior to the data collection time and the effect of Arake were assessed as dependent variable while other variables such weight, Renal function and liver function will manipulate to cause the effect (independent variables. i.e. avoiding/ restricting the mice from unnecessary meal and water intake other than recommended.

***Part III***: Taking and recording the every activity on daily basis during the study period. E.g Taking the weight once per week for individual mice using electronic beam balance (Kern and Sohn GmbH, D-72336, balingen Germany S.no 111316078), administering araqe and observing any behavioral change on the mice.

***Part IV***: Feeding of mice were provided after measured in Kg and discarding residual rodent chow left on the feeding cage also measured in Kg to evaluate food intake by the study animals and water for each group

***Part V***: Cage of each group of mice was cleaned using warm water and detergent daily and changed twice a week according to protocol to handle experimental laboratory animals.

***Part VI***: The experimental group was provided with distilled water and the experimental group will be treated with Arake concentration of 2ml, 4ml and 6ml/kg/bw at rate of 40% ethanol and after administration of Arake behavioral change were observed.

***Part VII***: At the end of the experiment the mice were sacrificed under anesthesia and neck to pubic incision will be done by experienced animal hander to take tissue sample (preserved in 10% NBF solution) and cardiac puncture was carried out to collect the blood for laboratory analysis

***Part VIII***: At the end of the data collection residual body part of the mice was discarded according to animal handling protocol to avoid environmental contamination.

***Part IX***: The collected tissue sample was processed for histopathological technique to prepare microscopic slides and determine the effect of local Araqe on the cardiac and hepatorenal structures.

***Part X***: Collected blood sample were further analyzed in laboratory to evaluated the effect of local arake on the hepatorenal functions

***Part XI***: The collected raw data from each study animal was recorded accordingly and entered into SPSS for further analysis.

***Part XII***: The analyzed data was presented in form of Mean and SEM, ANOVA test were used to compare the experimental and control groups.

## ANNEX III: Tissue processing steps

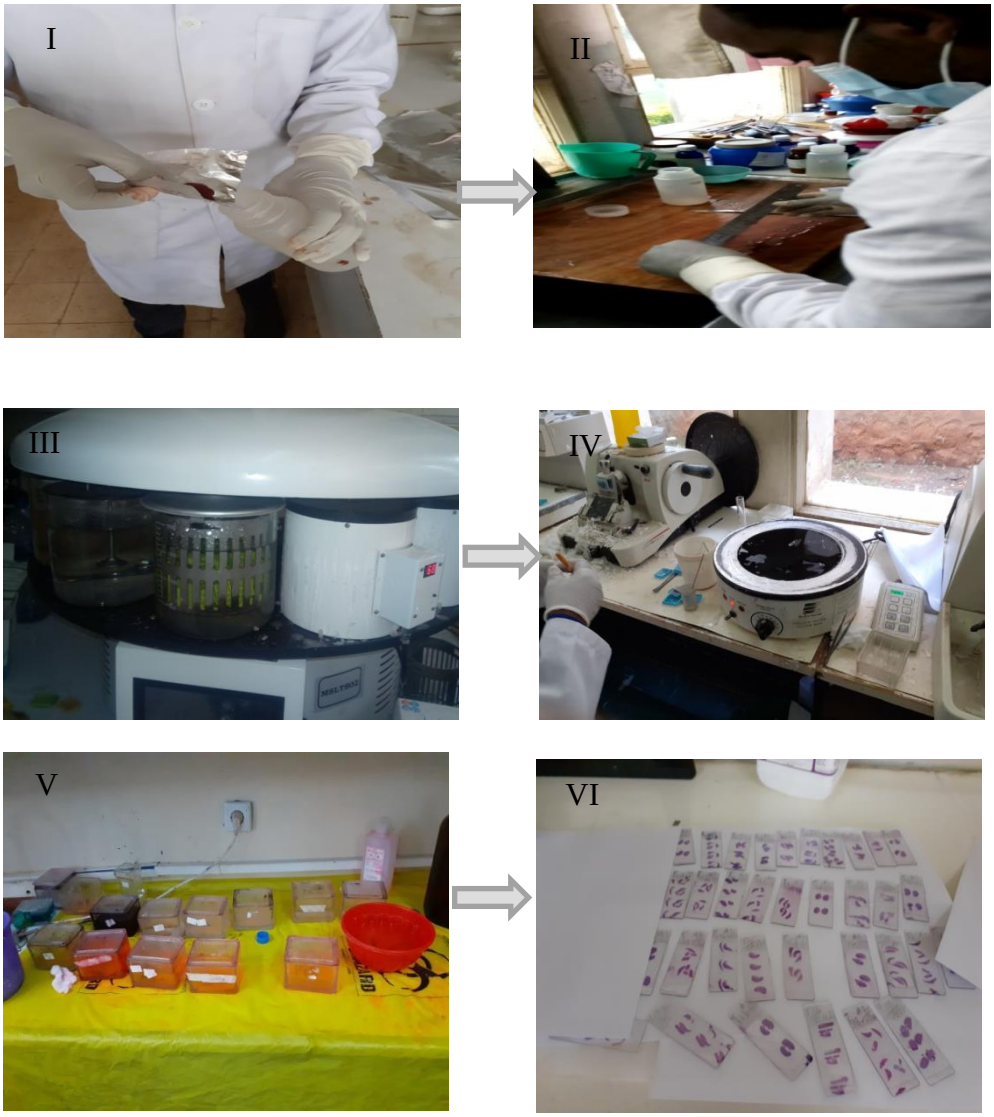


Fig 8: Shows steps of tissue processing for histopathological examination

**I**: Tissue preservation in 10% formalin solution **II**: Gross examination by pathologist

**III**: Tissue in tissue processing machine **IV**: Section of the tissue using rotatory microtome

**V**: Staining and staining solutions (H and E) **V**: prepared microscopic slides

## ANNEX-IV: Post Hoc Tukey Analysis Table

Table 8: Multiple comparison of body weight in relation to Arake consumption in mice

| Dependent Variable | (I)Group | (J) Group | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| First W | I | II | .667 | 1.691 | .979 | -4.07 | 5.40 |
| | | III | -.500 | 1.691 | .991 | -5.23 | 4.23 |
| | | IV | -1.167 | 1.691 | .900 | -5.90 | 3.57 |
| | II | I | -.667 | 1.691 | .979 | -5.40 | 4.07 |
| | | III | -1.167 | 1.691 | .900 | -5.90 | 3.57 |
| | | IV | -1.833 | 1.691 | .703 | -6.57 | 2.90 |
| | III | I | .500 | 1.691 | .991 | -4.23 | 5.23 |
| | | II | 1.167 | 1.691 | .900 | -3.57 | 5.90 |
| | | IV | -.667 | 1.691 | .979 | -5.40 | 4.07 |
| | IV | I | 1.167 | 1.691 | .900 | -3.57 | 5.90 |
| | | II | 1.833 | 1.691 | .703 | -2.90 | 6.57 |
| | | III | .667 | 1.691 | .979 | -4.07 | 5.40 |
| Second W | I | II | .667 | 1.599 | .975 | -3.81 | 5.14 |
| | | III | -.333 | 1.599 | .997 | -4.81 | 4.14 |
| | | IV | -2.333 | 1.599 | .479 | -6.81 | 2.14 |
| | II | I | -.667 | 1.599 | .975 | -5.14 | 3.81 |
| | | III | -1.000 | 1.599 | .923 | -5.47 | 3.47 |
| | | IV | -3.000 | 1.599 | .269 | -7.47 | 1.47 |
| | III | I | .333 | 1.599 | .997 | -4.14 | 4.81 |
| | | II | 1.000 | 1.599 | .923 | -3.47 | 5.47 |
| | | IV | -2.000 | 1.599 | .603 | -6.47 | 2.47 |
| | IV | I | 2.333 | 1.599 | .479 | -2.14 | 6.81 |
| | | II | 3.000 | 1.599 | .269 | -1.47 | 7.47 |
| | | III | 2.000 | 1.599 | .603 | -2.47 | 6.47 |
| Third W | I | II | .833 | 1.641 | .956 | -3.76 | 5.43 |
| | | III | -.333 | 1.641 | .997 | -4.93 | 4.26 |
| | | IV | -.333 | 1.641 | .997 | -4.93 | 4.26 |
| | II | I | -.833 | 1.641 | .956 | -5.43 | 3.76 |
| | | III | -1.167 | 1.641 | .892 | -5.76 | 3.43 |
| | | IV | -1.167 | 1.641 | .892 | -5.76 | 3.43 |
| | III | I | .333 | 1.641 | .997 | -4.26 | 4.93 |
| | | II | 1.167 | 1.641 | .892 | -3.43 | 5.76 |
| | | IV | .000 | 1.641 | 1.000 | -4.59 | 4.59 |
| | IV | I | .333 | 1.641 | .997 | -4.26 | 4.93 |
| | | II | 1.167 | 1.641 | .892 | -3.43 | 5.76 |
| | | III | .000 | 1.641 | 1.000 | -4.59 | 4.59 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Fourth W | I | II | 2.500 | 1.722 | .483 | -2.32 | 7.32 |
| | | III | 3.667 | 1.722 | .178 | -1.15 | 8.49 |
| | | IV | 1.667 | 1.722 | .769 | -3.15 | 6.49 |
| | II | I | -2.500 | 1.722 | .483 | -7.32 | 2.32 |
| | | III | 1.167 | 1.722 | .904 | -3.65 | 5.99 |
| | | IV | -.833 | 1.722 | .962 | -5.65 | 3.99 |
| | III | I | -3.667 | 1.722 | .178 | -8.49 | 1.15 |
| | | II | -1.167 | 1.722 | .904 | -5.99 | 3.65 |
| | | IV | -2.000 | 1.722 | .657 | -6.82 | 2.82 |
| | IV | I | -1.667 | 1.722 | .769 | -6.49 | 3.15 |
| | | II | .833 | 1.722 | .962 | -3.99 | 5.65 |
| | | III | 2.000 | 1.722 | .657 | -2.82 | 6.82 |
| Fifth W | I | II | 2.833 | 1.593 | .312 | -1.62 | 7.29 |
| | | III | 4.667* | 1.593 | .038 | .21 | 9.12 |
| | | IV | 3.333 | 1.593 | .189 | -1.12 | 7.79 |
| | II | I | -2.833 | 1.593 | .312 | -7.29 | 1.62 |
| | | III | 1.833 | 1.593 | .663 | -2.62 | 6.29 |
| | | IV | .500 | 1.593 | .989 | -3.96 | 4.96 |
| | III | I | -4.667* | 1.593 | .038 | -9.12 | -.21 |
| | | II | -1.833 | 1.593 | .663 | -6.29 | 2.62 |
| | | IV | -1.333 | 1.593 | .836 | -5.79 | 3.12 |
| | IV | I | -3.333 | 1.593 | .189 | -7.79 | 1.12 |
| | | II | -.500 | 1.593 | .989 | -4.96 | 3.96 |
| | | III | 1.333 | 1.593 | .836 | -3.12 | 5.79 |
| Sixth W | I | II | 4.333 | 1.598 | .060 | -.14 | 8.81 |
| | | III | 7.333* | 1.598 | .001 | 2.86 | 11.81 |
| | | IV | 6.500* | 1.598 | .003 | 2.03 | 10.97 |
| | II | I | -4.333 | 1.598 | .060 | -8.81 | .14 |
| | | III | 3.000 | 1.598 | .269 | -1.47 | 7.47 |
| | | IV | 2.167 | 1.598 | .540 | -2.31 | 6.64 |
| | III | I | -7.333* | 1.598 | .001 | -11.81 | -2.86 |
| | | II | -3.000 | 1.598 | .269 | -7.47 | 1.47 |
| | | IV | -.833 | 1.598 | .953 | -5.31 | 3.64 |
| | IV | I | -6.500* | 1.598 | .003 | -10.97 | -2.03 |
| | | II | -2.167 | 1.598 | .540 | -6.64 | 2.31 |
| | | III | .833 | 1.598 | .953 | -3.64 | 5.31 |

*. The mean difference is significant at the 0.05 level.

Table 9: Multiple comparison of serum level of AST and Alt as Liver function biomarkers in relation to Arake consumption in mice

| Dependent Variable | (I) Group | (J) Group | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| AST | I | II | -44.7333333* | 13.0095414 | .013 | -81.146210 | -8.320457 |
| | | III | -126.8500000* | 13.0095414 | .000 | -163.262877 | -90.437123 |
| | | IV | -135.4166667* | 13.0095414 | .000 | -171.829543 | -99.003790 |
| | II | I | 44.7333333* | 13.0095414 | .013 | 8.320457 | 81.146210 |
| | | III | -82.1166667* | 13.0095414 | .000 | -118.529543 | -45.703790 |
| | | IV | -90.6833333* | 13.0095414 | .000 | -127.096210 | -54.270457 |
| | III | I | 126.8500000* | 13.0095414 | .000 | 90.437123 | 163.262877 |
| | | II | 82.1166667* | 13.0095414 | .000 | 45.703790 | 118.529543 |
| | | IV | -8.5666667 | 13.0095414 | .911 | -44.979543 | 27.846210 |
| | IV | I | 135.4166667* | 13.0095414 | .000 | 99.003790 | 171.829543 |
| | | II | 90.6833333* | 13.0095414 | .000 | 54.270457 | 127.096210 |
| | | III | 8.5666667 | 13.0095414 | .911 | -27.846210 | 44.979543 |
| ALT | I | II | -40.5916667* | 11.7394805 | .012 | -73.449724 | -7.733609 |
| | | III | -26.0250000 | 11.7394805 | .153 | -58.883057 | 6.833057 |
| | | IV | -50.2083333* | 11.7394805 | .002 | -83.066391 | -17.350276 |
| | II | I | 40.5916667* | 11.7394805 | .012 | 7.733609 | 73.449724 |
| | | III | 14.5666667 | 11.7394805 | .609 | -18.291391 | 47.424724 |
| | | IV | -9.6166667 | 11.7394805 | .845 | -42.474724 | 23.241391 |
| | III | I | 26.0250000 | 11.7394805 | .153 | -6.833057 | 58.883057 |
| | | II | -14.5666667 | 11.7394805 | .609 | -47.424724 | 18.291391 |
| | | IV | -24.1833333 | 11.7394805 | .200 | -57.041391 | 8.674724 |
| | IV | I | 50.2083333* | 11.7394805 | .002 | 17.350276 | 83.066391 |
| | | II | 9.6166667 | 11.7394805 | .845 | -23.241391 | 42.474724 |
| | | III | 24.1833333 | 11.7394805 | .200 | -8.674724 | 57.041391 |

*. The mean difference is significant at the 0.05 level.

Table 10: Multiple comparison of BUN and SCRt as renal function biomarkers in relation to Arake consumption in mice

| Dependent Variable | (I) Group | (J) Group | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| BUN | I | II | -24.833* | 8.074 | .028 | -47.43 | -2.23 |
| | | III | -41.000* | 8.074 | .000 | -63.60 | -18.40 |
| | | IV | -54.167* | 8.074 | .000 | -76.77 | -31.57 |
| | II | I | 24.833* | 8.074 | .028 | 2.23 | 47.43 |
| | | III | -16.167 | 8.074 | .220 | -38.77 | 6.43 |
| | | IV | -29.333* | 8.074 | .008 | -51.93 | -6.73 |
| | III | I | 41.000* | 8.074 | .000 | 18.40 | 63.60 |
| | | II | 16.167 | 8.074 | .220 | -6.43 | 38.77 |
| | | IV | -13.167 | 8.074 | .385 | -35.77 | 9.43 |
| | IV | I | 54.167* | 8.074 | .000 | 31.57 | 76.77 |
| | | II | 29.333* | 8.074 | .008 | 6.73 | 51.93 |
| | | III | 13.167 | 8.074 | .385 | -9.43 | 35.77 |
| SCRt | I | I | -.167 | .190 | .817 | -.70 | .37 |
| | | III | -.333 | .190 | .324 | -.87 | .20 |
| | | IV | -.333 | .190 | .324 | -.87 | .20 |
| | II | I | .167 | .190 | .817 | -.37 | .70 |
| | | III | -.167 | .190 | .817 | -.70 | .37 |
| | | IV | -.167 | .190 | .817 | -.70 | .37 |
| | III | I | .333 | .190 | .324 | -.20 | .87 |
| | | II | .167 | .190 | .817 | -.37 | .70 |
| | | IV | .000 | .190 | 1.000 | -.53 | .53 |
| | IV | I | .333 | .190 | .324 | -.20 | .87 |
| | | II | .167 | .190 | .817 | -.37 | .70 |
| | | III | .000 | .190 | 1.000 | -.53 | .53 |

*. The mean difference is significant at the 0.05 level.

## ANNEX V: Ethical approval of research protocol by institutional review board

**Jimma University Institute of Health**

**Institutional Review Board**

Ref.No IHRPGD/835/20

Date: 5/10/2020

To: Rebuma Sorsa:

**Subject: Ethical Approval of Research Protocol**

The IRB of Institute of Health has reviewed your research project **"Effect of Arake on Hepatorenal Structures and Functions in Swiss Albino Mice: Lab-Based Acute Toxicity Study".**

Thus, this is to notify that your/this research protocol has presented to the IRB meets the ethical and scientific standards outlined in national and international guidelines. Hence, we are pleased to inform you that your research protocol is ethically cleared.

We strongly recommend that any significant deviation from the methodological details indicated in the approved protocol must be communicated to the IRB before it has been implemented.

With Regards!

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**DECLARATION**

This is to certify that the thesis prepared by Rebuma Sorsa entitled **Effect of local Arake on hepatorenal structures and functions in Swiss Albino Mice** and submitted in the partial fulfillment of the requirements for Degree of Master science in Clinical Anatomy complies with regulation of Jimma University and I undersigned agrees to accept responsibility for the scientific ethical, originality, quality and technical conduct of this research provision of required progress reports.

Name of the student: _____
Date _____ Signature _____

**Approval of Advisor(s)**

1. Tilahun A. Nigatu (Assistant Professor of Human Anatomy)
   Signature _____ Date _____
2. Niguse Hamba (MSc in Human Anatomy)
   Signature _____ Date _____
3. Dr. Dechasa Tegegne (DVM, MSc in Animal Biotechnology)
   Signature _____ Date _____

Dr.Gebi Namo (MD, Pathologist) Signature _____ Date _____

**Approval of examiner(s)**

Name of examiner: _____
Date _____ Signature _____

**Approval of school/department head**

Name of school/department head: _____
Date _____ Signature _____