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Histomorphological Assessment and Characterization of The Alcoholic Extract of *Azadirachta Indica* Heartwood as A Counterstain

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Abstract

Azadirachta indica (Neem tree) belongs to the family Meliaceae which is found in abundance in tropical and semitropical regions like Nigeria. It is a fast-growing tree with 20–23 m tall and trunk is straight and has a diameter around 4-5 ft. The leaves are compound, imparipinnate, with each comprising 5–15 leaflets. This study was carried out to find out histomorphological assessment and characterization of the alcoholic extract of *Azadirachta indica* heartwood as a counterstain. The alcoholic extraction and characterization of the extract were conducted using maceration method and GC-MS analysis respectively. The extract was used to stain some selected organs (liver and kidney) at varying pH (4-6) and concentration (0.5%, 1% and 2%) for the duration of 2 minutes, 3 minutes and 5 minutes to established staining interactions of the alcoholic extract. The data were presented as tables, graph and photomicrographs. The selected organs gave better staining properties with 0.5%, and 1% concentration at all the pH variation and time interval used. And also revealed the presence of many bioactive compounds like (Methylthio)-acetonitrile, 1-(2'-Hydroxy-5'-methylphenyl)-1propanone (E)-oxime, cis-4-Ethoxy-b-methyl-b-nitrostyrene, 2-formylCyclopropanecarboxamide, N-benzoyl oxy-, 4-(4-methylphenyl)-5-phenoxy-6-phenyl-, 4-(4-methylphenyl)-5-phenoxy-6-phenyl-, 1-Iodo-2-methylnonane, 2,3-bis (phenyl amino)-, 3-(4-nitrophenyl)-1-phenyl- etc. This study established the cytoplasmic counter-staining ability of Azadirachta *indica* heartwood extract solution and it is therefore suggested that alcoholic extract (0.5% and 1%) of *Azadirachta indica* can be substituted for eosin due to its domestic availability and ease of preparation.

Keywords: *Azadirachta indica,* Counterstain, Extraction, Characterization, Tissues

Introduction

Azadirachta indica (Neem tree) belongs to the family Meliaceae which is found in abundance in tropical and semitropical regions like Nigeria. It is a fast-growing tree with 20–23 m tall and trunk is straight and has a diameter around 4-5 ft. The leaves are compound, imparipinnate, with each comprising 5–15 leaflets *Azadirachta indica* (Neem tree). The commercially important part of the plant is the fruits, seeds, oil, leaves and back. It also has a medicinal value (antimicrobial effect and anti-inflammatory) and cosmetics (Debjit *et al*., 2010). The plant is widely grown in Northern Nigeria and it is commonly known as dogonyaro in Hausa. The heartwood part is mottled and pinkish red in colour when exposed. It is very strong, durable, and bitter in taste (Ojigbo *et al.,* 2013).

Figure 1: *Azadirachta indica* Plant. A: heartwood and sapwood; B: Flowers and leaves (https://garden.org/neem tree plant, 2010).

Stains: Stains have been used to enhance accurate descriptions of the microscopic structure of tissues, which is necessary for histopathology diagnosis (Egbujo *et al*., 2008). If unstained tissue sections are viewed under microscope, the refractive index, structure and component of tissue are ambiguous. However, when stained tissue sections are examined under microscope, the structure becomes well appreciated, and components of the tissue are specifically defined (Bancroft *et al*., 2012). Histological stains are substance or biological dyes which colour tissue in order to enhance optical differentiation of tissue component (Avwioro *et al*., 2014). Dyes are colored substance which impinges colour for material to enhance optical differentiation such as textile, cosmetic, food, drugs, rubber, plastics, tissues and hair (Bhuyan *and Saikia.,* 2005). Stains also refer to as dyes which have affinity to a particular component of the cell (Ochei and Kolhathar, 2007). Some dyes reagents require the addition of mordant, oxidants, accelerators and adjustment of pH before they can stain tissues while others do not require these substances for them to stain the tissue or other tissue components. Mordant act as a bridge between the dye and the tissue, facilitator improve the quality of the staining, while, accentuator and accelerator increase staining power of dyes to an ideal level (Ochei, 2007). The colour of stained tissue section depends on the nature of the chromophores, the substituent functional groups, and the auxochromes of the dye molecular species. Chromophores and auxochromes are considered the most important chemical constituents of the dyes responsible for coloration. Dye-yielding plants, unlike synthetic dyes, may contains more than one chemical constituent, each contains a different colour and properties, function singly or in combination with the different group, depending on their chemical composition and structure (Ochei and Kolhatkar, 2007). In Africa particularly in Nigeria there are uncountable naturally occurring dye plants, which are tillable, just as they are cultivated in other countries around the globe. Recent studies have given useful results about such plants that can be used as histological stains for some tissues components identification (Avwioro *et al*., 2005). This is considering the significant amount of foreign exchange and process involved in obtaining suitable synthetic dye for histological purposes, and also to their hazardous effects to human and animals' health (Avwioro *et al*., 2005). This has resulted in withdrawal of some dyes as their harmful effects to human and animals become addressed (Bhuyan and Saikia, 2005). Therefore, greater attention and effort are now channeled toward the use of natural dyes extracted from plant, which is less expensive than costly synthetic dyes (Avwioro *et al*., 2005). This study was conduct to determine the

histomorphological assessment and characterization of the alcoholic extract of *Azadirachta indica* heartwood as a counterstain on some selected animal tissues.

Material and Methods

Study Location

The study was conducted at the Department of Histopathology, School of Medical Laboratory Sciences and Department of Pharmacognosy and Ethno Pharmacy, Faculty of Pharmaceutical science, Usmanu Danfodiyo University, Sokoto.

Plant Identification

The plant taxonomic identification and assigning of specimen Voucher Number was carried out in the Department of Pharmacognosy and Ethno Pharmacy, Faculty of Pharmaceutical Science, Usmanu Danfodiyo University, Sokoto state. And the Voucher number was given as PCG/UDUS/MELI/0001.

Ethical Consideration

Ethical approval for this research was obtained from Research and Ethical Committee, Department of Pharmacognosy and Ethno Pharmacy, Faculty of Pharmaceutical Science, Usmanu Danfodiyo University, Sokoto State.

Procurement of *Azadirachta indica* Heartwood and Wister rats

The dried red calyx of *Azadirachta indica* (neem tree) heartwood was purchased from Gobir central market Sabo Birnin Local Government, Sokoto State. The Wistar rats was procured from the animal house of Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Skoto State.

Processing of Tissues

Three Wistar rats were anaesthetized using chloroform vapour in an enclosed transparence jar and longitudinal abdominal incision were made and the organs such liver and kidney were carefully harvested and saline washed fixed in 10% formal saline for 48 hours. The tissues were dehydrated, cleared, impregnated, embedded and sectioned to produce the microscopic slides sections liver and kidney.

Table 2: Experimental Design

Extract (G)	Glacial Acetic Acid (0.5ml)	Ammonium Hydroxide (0.5ml)	PH Reaction	Distilled Water (ml)	Duration (Minutes)
0.5g	0.5ml	_	6	upto100ml	5
0.5g	2ml	_	5	upto100ml	3
0.5g	4m	0.5ml	4	upto100ml	2
1g	0.5ml	_	6	upto100ml	5
1g	2ml	_	5	upto100ml	3
1g	4m	0.5ml	4	upto100ml	2
2g	_		6	upto100ml	5
2g	_	_	5	upto100ml	3
2g	_	0.5ml	4	upto100ml	2

Alcoholic Extraction Procedure

i. The *Azadirachta indica* heartwood was grinded into powder with mortar and pestle.
ii. About five hundred grams (500g) of the powder was weighed with digital weighing balance and transferred in to the 2000 ml capacity conical flask.
iii. About 1000ml of absolute alcohol was added into the flask containing 500g of powdered *Azadirachta indica* heartwood, mixed and allowed to stand for 24 hours.
iv. After 24 hours, the filtrate was obtained using filter paper and was placed in hot air oven at 60℃ for three days to completely get rid of the solvent.

Acidic Preparation

i. The extract was acidified by addition of 0.5ml of glacial acetic acid until a desired pH was obtained.
ii. The extract was modified to pH closed to 7 by adding 0.5ml of ammonium hydroxide to 99.5ml of the extract.

Procedure for Standard Haematoxylin and Eosin Staining Technique

i. Sections were taken to water.
ii. Sections were then stained in Mayer Haematoxylin for 5 minutes.
iii. They were then washed in running tap water for 2 minutes
iv. Sections were differentiated in 1% acid alcohol for a few seconds
v. They were then washed in running tap water (blued) for 5 minutes
vi. Tissue sections were stained in 1% aqueous eosin for 2 minutes.
vii. Sections were then washed in water
viii. Finally, tissue sections were properly dehydrated, cleared and mounted using distyrene plasticizer and xylene (DPX) and examined under microscope.

Procedure for Haematoxylin and *Azadirachta indica* Staining Technique

i. Sections were taken to water.
ii. Sections were then stained in Mayer hematoxylin for 5 minutes.
iii. They were then washed in running tap water for 2 minutes
iv. Sections were differentiated in 1% acid alcohol for a few seconds
v. They were then washed in running tap water (blued) for 5 minutes
vi. Tissue sections were counterstained with *Azadirachta indica* solution of various concentrations (0.5%, 1%, 2 %,) under varying time duration (2min, 3min and 5min) and pH (4-6).
vii. Sections were then washed in water
viii. Finally, tissue sections were properly dehydrated, cleared and mounted using distyrene plasticizer and xylene (DPX) and examined under microscope.

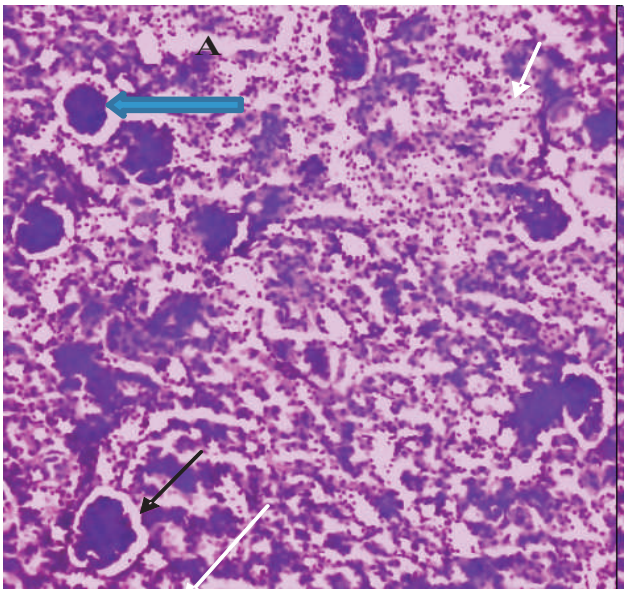
GC-MS Analysis

GC-MS analysis was carried out on Agilent Technologies Intuvo 9000 GC System and Agilent Technologies 5977B Mass Selective Detector (MSD) coupled with 4513A Automatic Liquid Sampler (ALS). The part number of the column used was Agilent 1909IS – 483UI – INT capillary column with the specification HP – 5MS UI 30m, 0.25mm, 0.25µm.

Statistical Analysis

The data obtained were presented in tables, graphs and photomicrographs.

Results



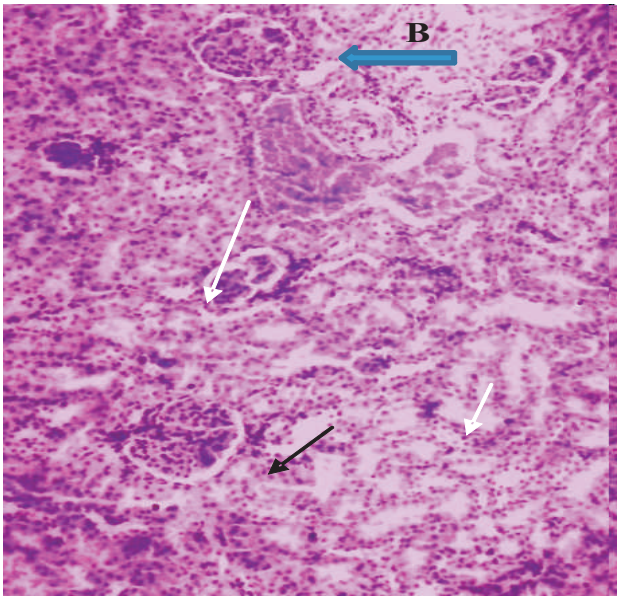
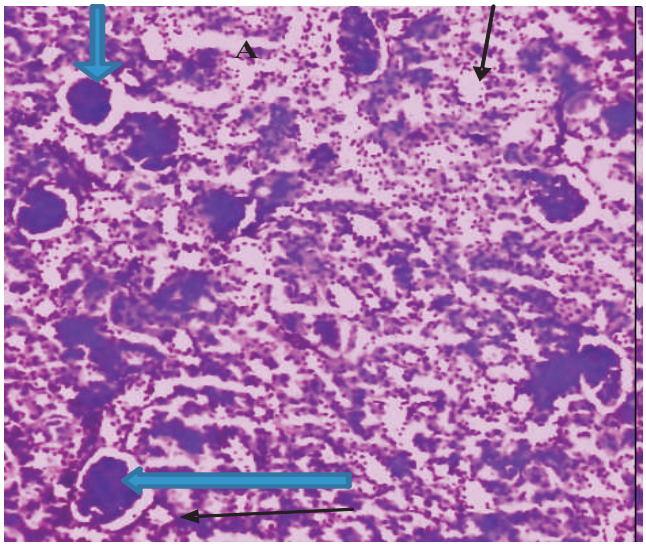


Plate 1A: A photomicrograph of kidney section obtained from staining with H and E as control (x100).
Plate 1B: A photomicrograph of kidney section obtained from staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 4 for 2 minutes (x100).

Legend
Blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, black arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short white arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long white arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B.**



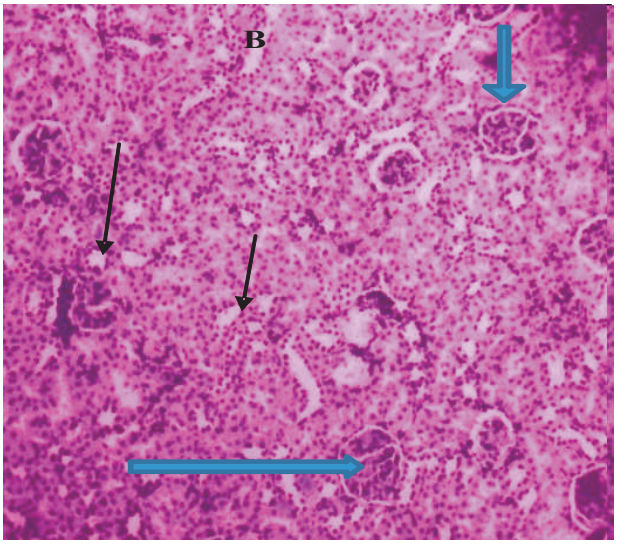
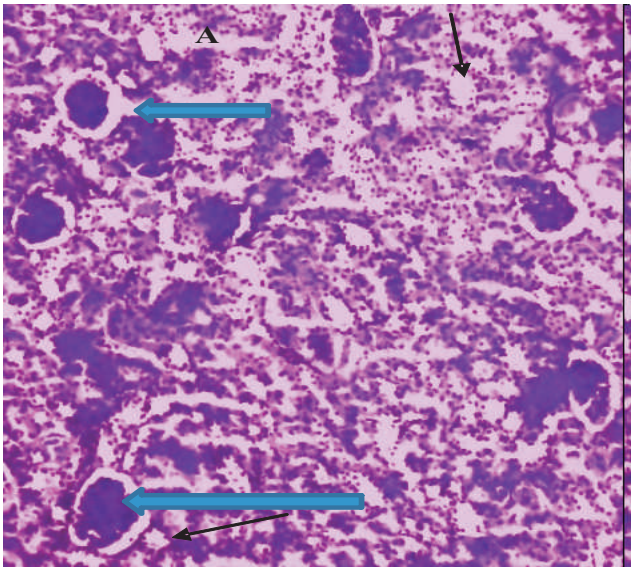


Plate 2A: A photomicrograph of kidney obtained from staining with H and E as control (x100).
Plate 2B: A photomicrograph of kidney obtained from staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 5 for 3 minutes (x100).

Legend
Long blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, short blue arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.



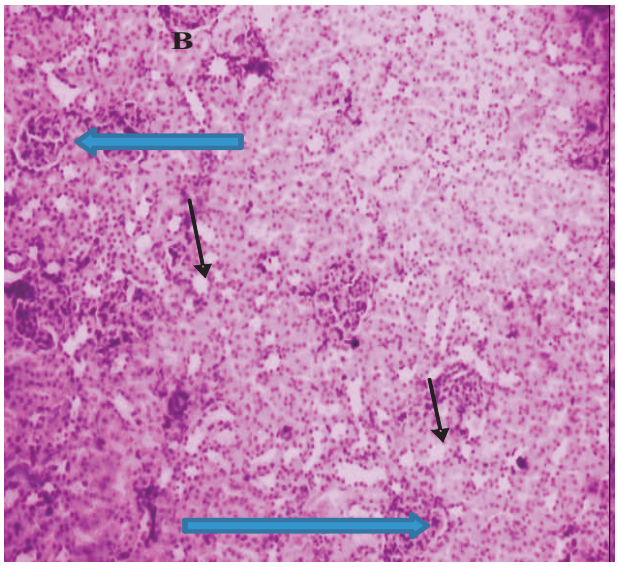


Plate 3A: A photomicrograph of kidney obtained from staining with H and E as a control (x100).
Plate 3B: A photomicrograph of kidney obtained from staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 6 for 5 minutes (x100).

Legend
Long blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, short blue arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

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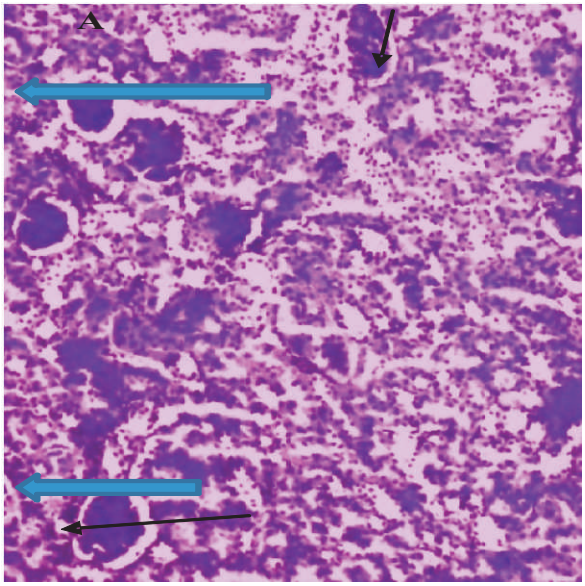

2.0

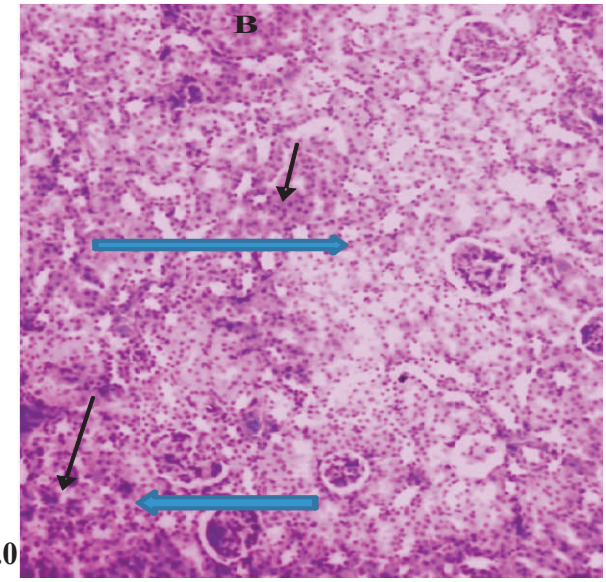

Plate 4A: A photomicrograph of kidney obtained from staining with H and E as a control (x100).
Plate 4B: A photomicrograph of kidney obtained from staining with haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 4 for 2minutes (x100).

Legend
Short blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, long blue arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

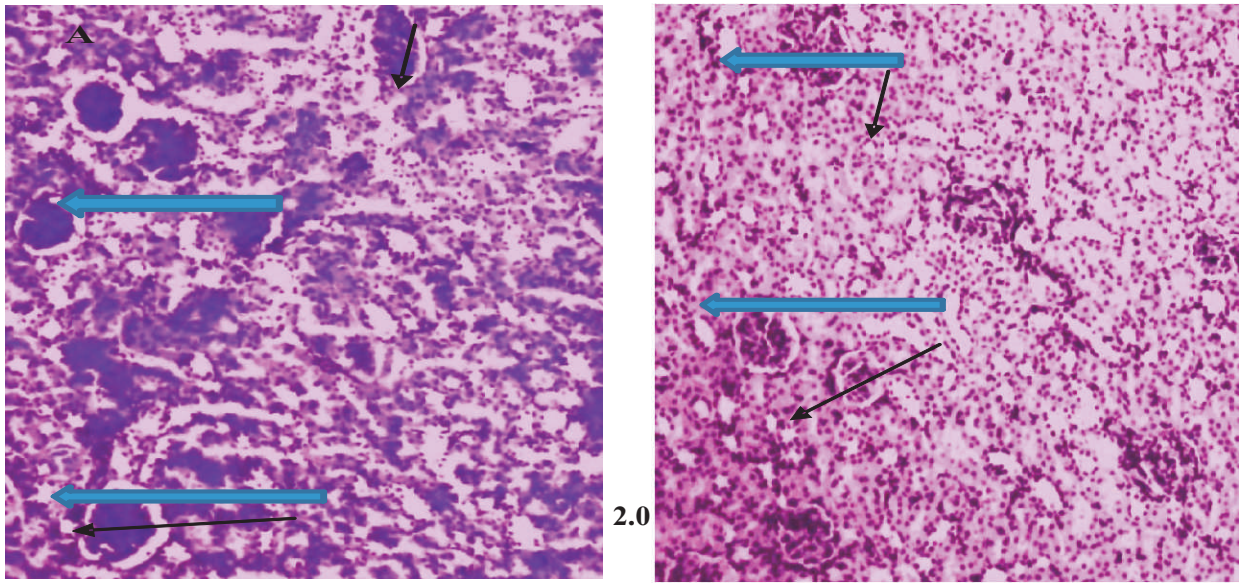

Plate 5A: A photomicrograph of kidney obtained from staining with H and E as a control (x100).
Plate 5B: A photomicrograph of kidney obtained from staining with haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 5 for 3minutes (x100).

Legend
Short blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, long blue arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

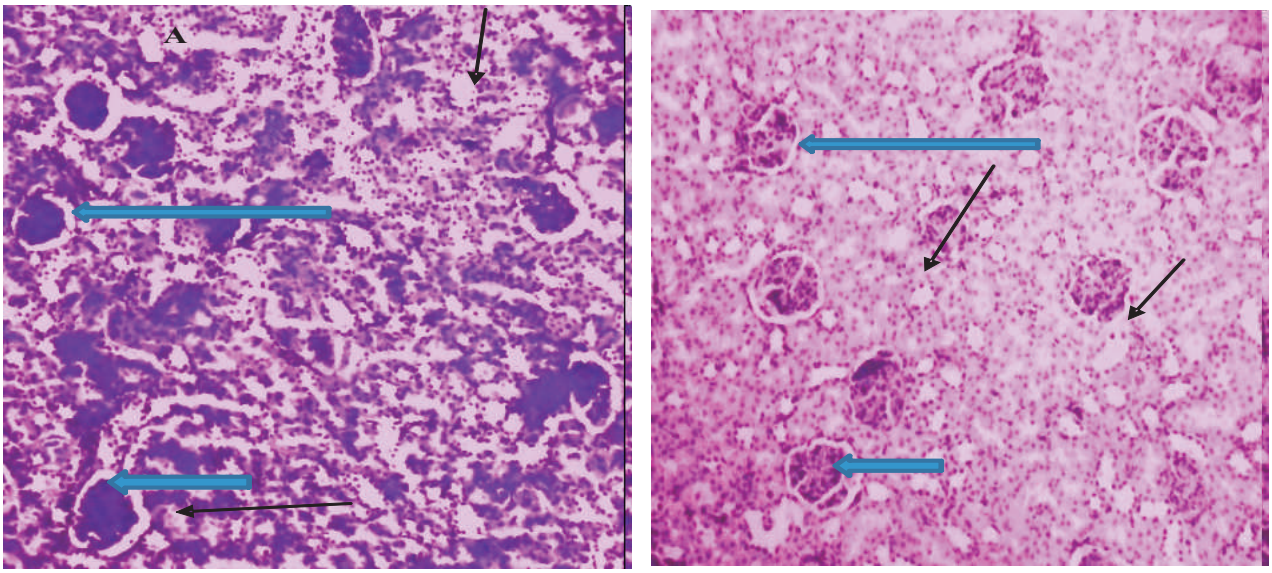

Plate 6A: A photomicrograph of kidney obtained from staining with H and E as a control (x100).
Plate 6B: A photomicrograph of kidney obtained from staining with haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 6 for 5 minutes (x100).

Legend
Short blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, long blue arrow: This showing well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing c well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

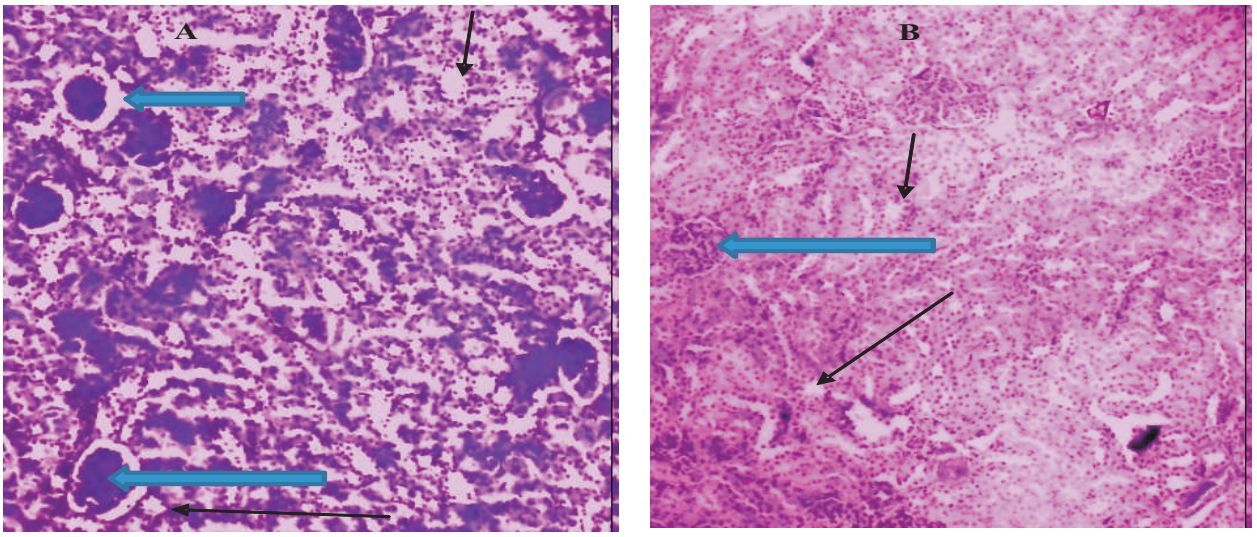

Plate 7A: A photomicrograph of kidney obtained from staining with H and E as control (x100).
Plate 7B: A photomicrograph of kidney obtained from staining with haematoxylin and 2% *Azadirachta indica heartwood* extract solution at pH 4 for 2 minutes (x100).

Legen
Long blue arrow: This showing fairly demonstrated glomerulus on plate **B** while clearly demonstrated on plate **A**, short blue arrow: This showing poorly demonstrated bowman's capsule on plate **B** while well demonstrated on plate **A,** short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

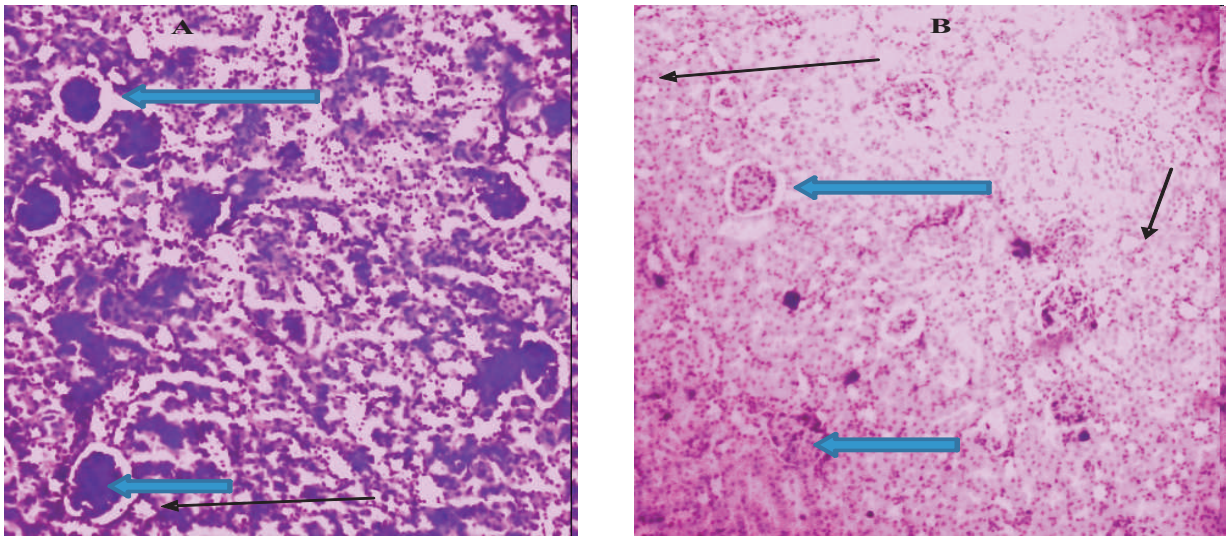

Plate 8A: A photomicrograph of kidney obtained from staining with H and E as a control (x100).
Plate 8B: A photomicrograph of kidney obtained from staining with haematoxylin and 2% *Azadirachta indica heartwood* extract solution at pH 5 for 3 minutes (x100).

Legend
Short blue arrow: This showing fairly demonstrated glomerulus on plate **B** while clearly demonstrated on plate **A**, long blue arrow: This showing poorly demonstrated bowman's capsule on plate **B** while well demonstrated on plate **A,** short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A** and **B**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A** and **B**.

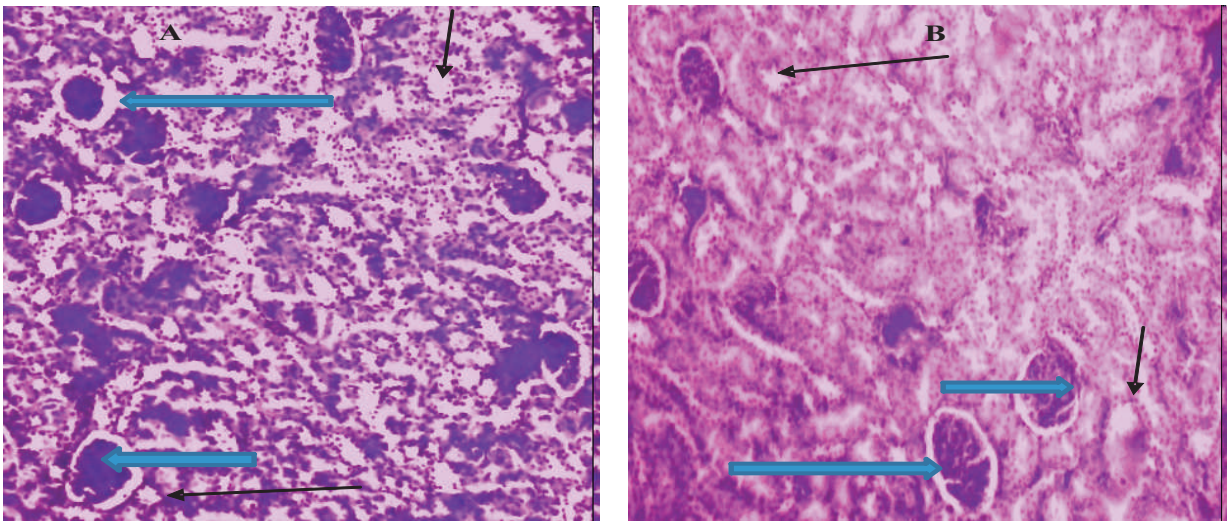

Plate 9A: A Photomicrograph of kidney obtained from staining with H and E as a control (x100). Plate 9B: A Photomicrograph of kidney obtained from staining with haematoxylin and 2% *Azadirachta indica heartwood* extract solution at pH 6 for 5 minutes (x100).

Legend

Short blue arrow: This showing well demonstrated glomerulus on both plate **A** and **B**, long blue arrow: This showing c well demonstrated bowman's capsule on both plate **A** and **B**, short black arrow: This showing well demonstrated distal convoluted tubule on both plate **A**, and long black arrow: This showing well demonstrated proximal convoluted tubule on both plate **A**.

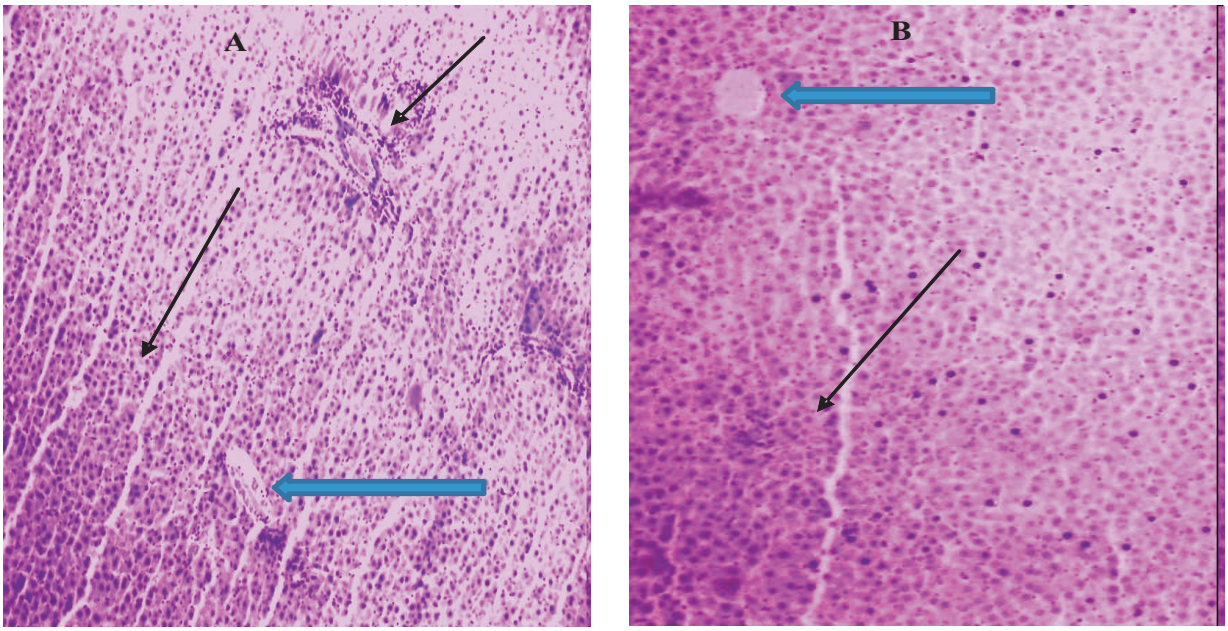

Plate 10A: A photomicrograph of liver section obtained from staining with H and E as a control (x100). Plate 10B: A photomicrograph of liver section obtained from staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 4 for 2 minutes (x100).

Legend

Long black arrow: This showing fairly demonstrated hepatocyte on plate **B** while clearly demonstrated on plate **A**, short black arrow: This showing well demonstrated portal triad on plate **A**, and blue arrow: This showing well demonstrated central vein on both plate **A** and **B.**

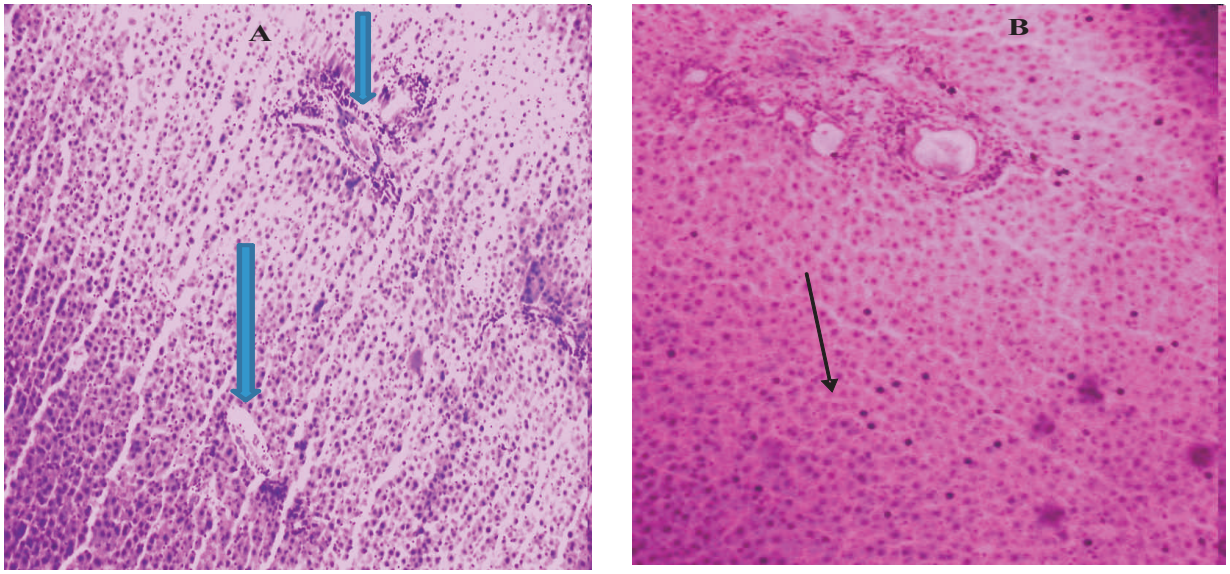

Plate 11A: A Photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 11B: A Photomicrograph of liver obtained from staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 5 for 3 minutes (x100).

Legend
Black arrow: This showing well demonstrated hepatocyte on Plate **A** while poorly demonstrated on Plate **B**, short blue arrow: This showing well demonstrated portal triad on Plate **A,** and long blue arrow: This showing well demonstrated central vein on Plate **A**.

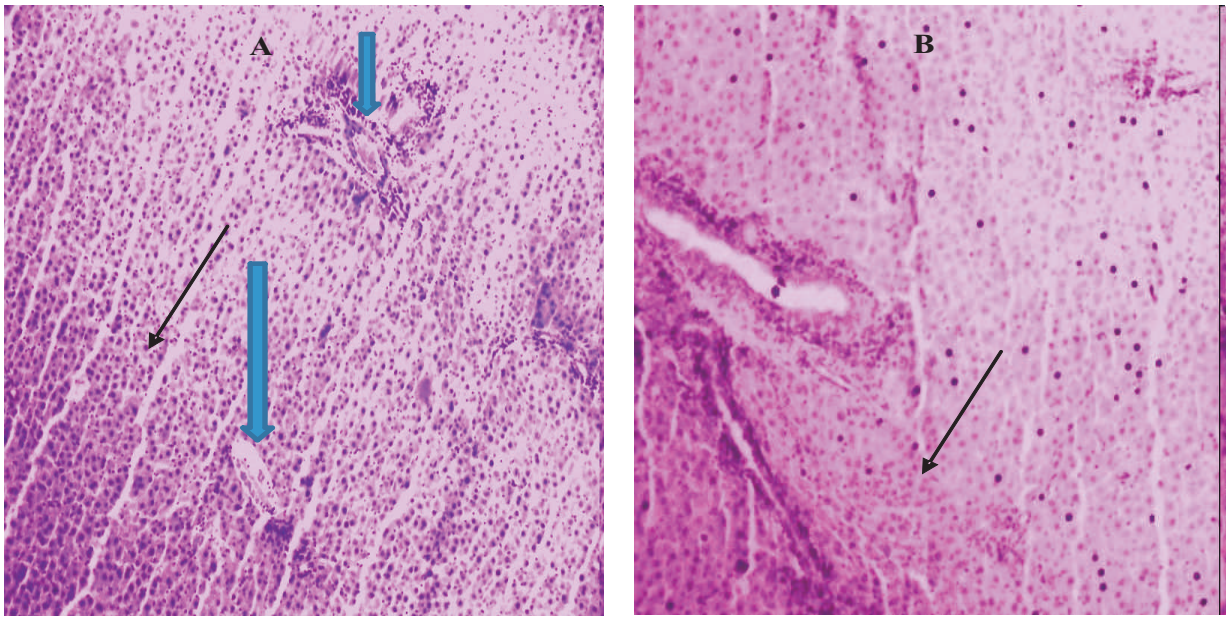

Plate 12A: A photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 12B: A photomicrograph of liver obtained from staining with staining with haematoxylin and 0.5% *Azadirachta indica heartwood* extract solution at pH 6 for 5 minutes (x100).

Legend
Black arrow: This showing clearly demonstrated hepatocyte on Plate **A** while poorly demonstrated on Plate **B,** short blue arrow: This showing well demonstrated portal triad on Plate **A,** and long blue arrow: This showing well demonstrated central vein on Plate **A.**

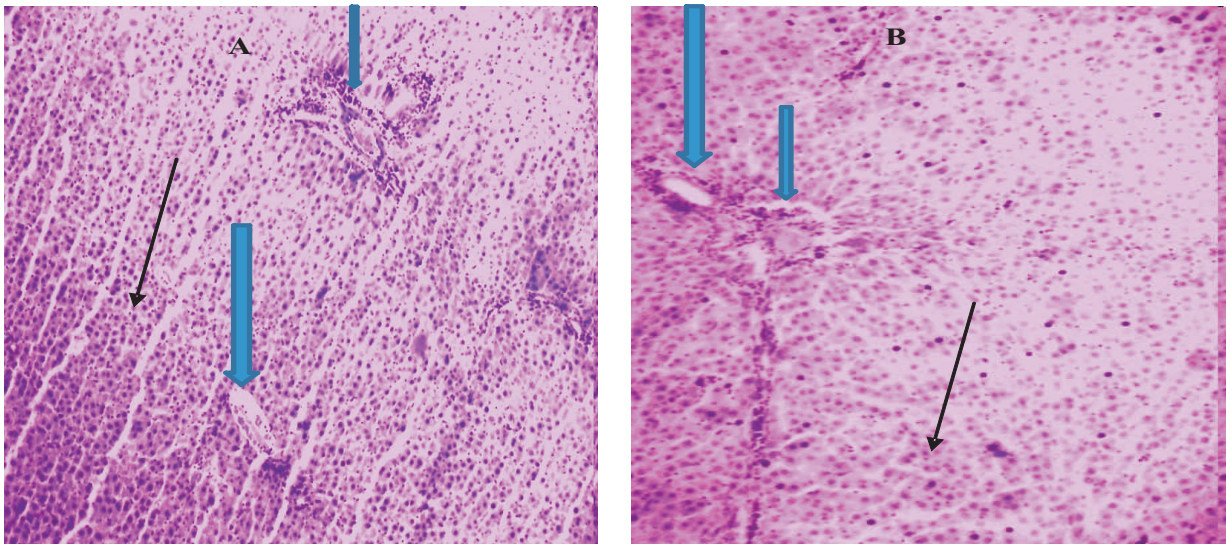

Plate 13A: A photomicrograph of liver obtained from staining with H and E as control (x100).
Plate 13B: A photomicrograph of liver obtained from staining with haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 4 for 2 minutes (x100).

Legend
Black arrow: This showing clearly demonstrated hepatocyte on Plate **A** while poorly demonstrated on Plate **B,** short blue arrow: This showing well demonstrated portal triad on Plate **A** while fairly demonstrated on Plate **B,** and long blue arrow: This showing well demonstrated central vein on Plate **A** while fairly demonstrated on Plate **B.**

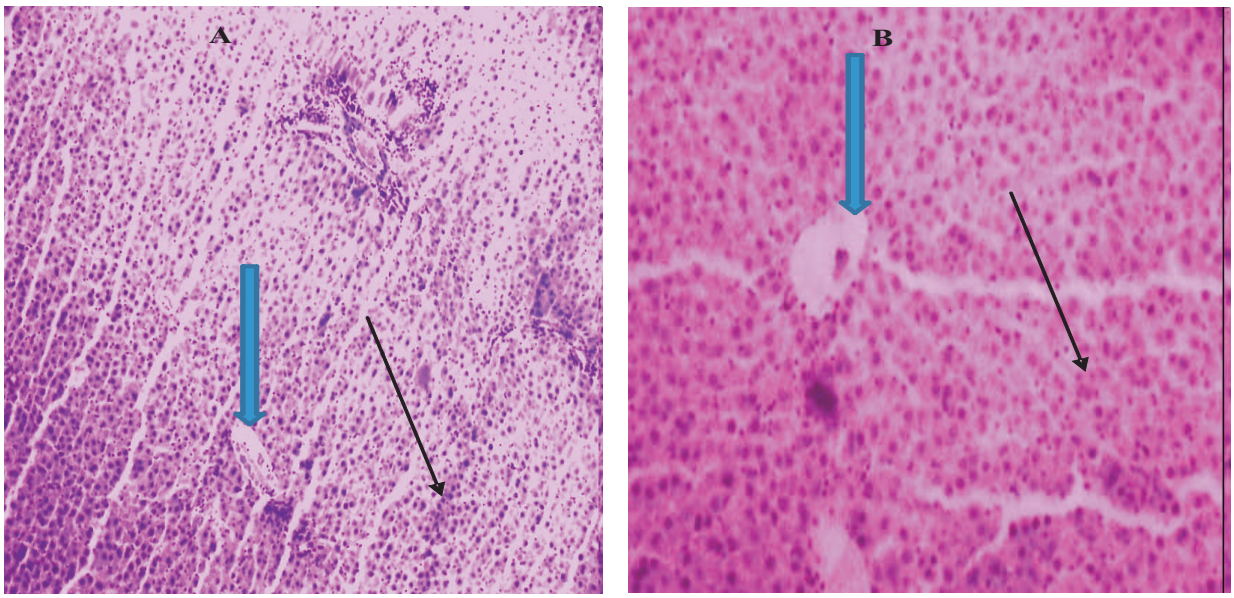

Plate 14A: A photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 14B: A photomicrograph of liver obtained from staining haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 5 for 3 minutes (x100).

Legend
Black arrow: This showing clearly demonstrated hepatocyte on Plate **A** while poorly demonstrated on Plate **B,** long blue arrow: This showing well demonstrated central vein on Plate **A** while fairly demonstrated on Plate **B,** and short blue arrow: This showing well demonstrated portal triad on Plate **A.**

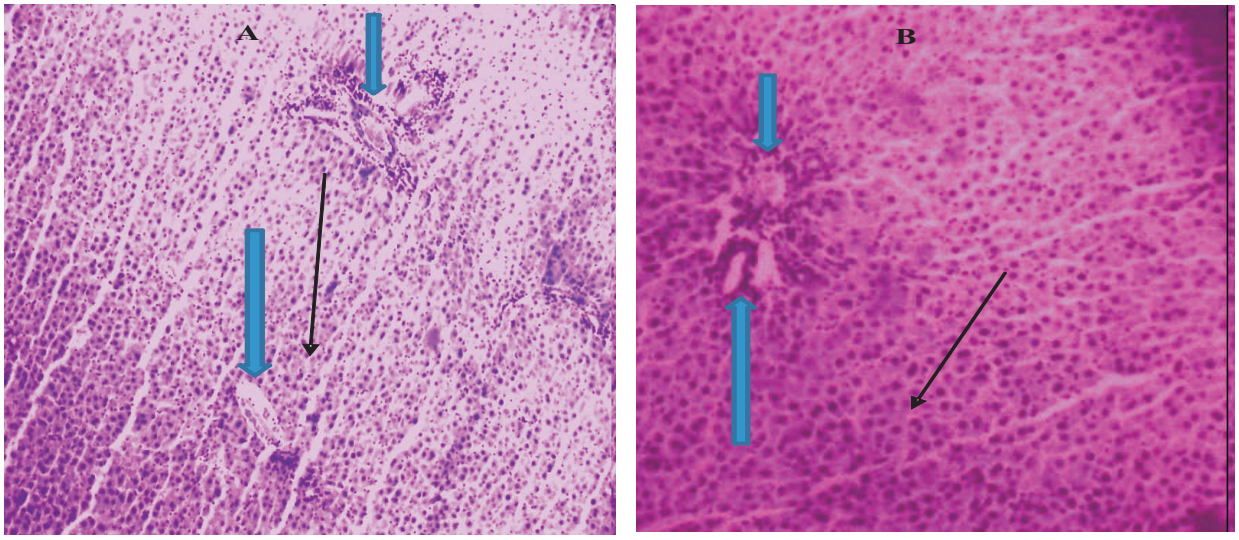

Plate 15A: A photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 15B: A photomicrograph of liver obtained from staining with staining with haematoxylin and 1% *Azadirachta indica heartwood* extract solution at pH 6 for 5 minutes (x100).

Legend
Black arrow: This showing clearly demonstrated hepatocyte on Plate **A** while fairly demonstrated on Plate **B,** long blue arrow: This showing well demonstrated central vein on Plate **A** while fairly demonstrated on Plate **B,** and short blue arrow: This showing well clearly demonstrated portal triad on Plate **A,** while fairly demonstrated on plate **B**.

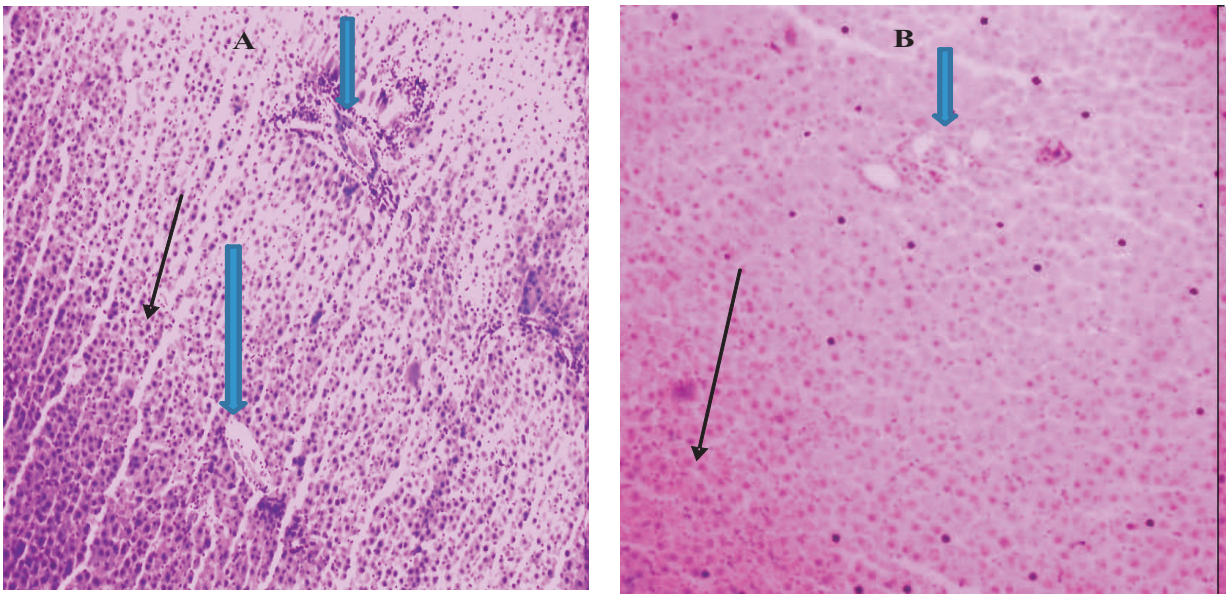

Plate 16A: A photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 16B: A photomicrograph of liver obtained from staining with haematoxylin and 2% *Azadirachta indica heartwood* extract solution at pH 4 for 2 minutes (x100).

Legend
Long blue arrow: This showing clearly demonstrated central vein on Plate **A,** short blue arrow: This showing well demonstrated portal triad on Plate **A,** while fairly demonstrated on plate **B**, and black arrow: This showing well demonstrated hepatocyte on Plate **A** while poorly demonstrated on Plate **B.**

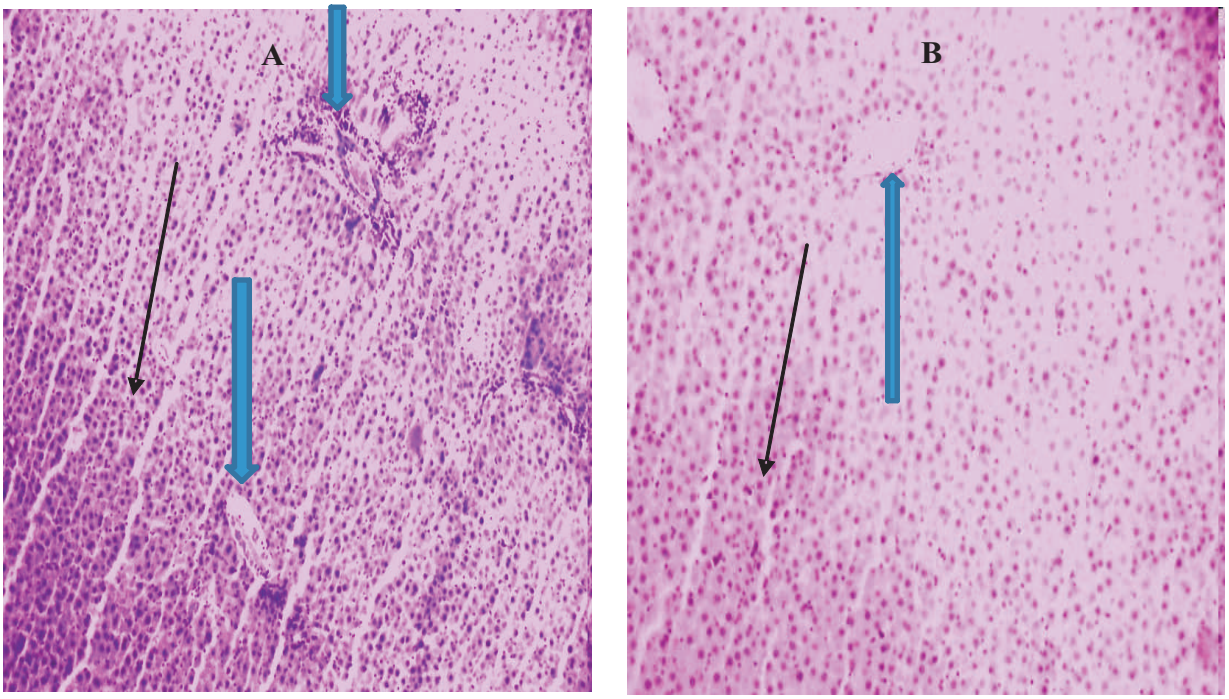

Plate 17A: A photomicrograph of liver obtained from staining with H and E as a control (x100).
Plate 17B: A photomicrograph of liver obtained from staining with haematoxylin and 2% *Azadirachta indica heartwood* extract solution at pH 5 for 3 minutes (x100).

Legend
Long blue arrow: This showing clearly demonstrated central vein on Plate **A** while fairly demonstrated on Plate **B,** black arrow: This showing well demonstrated hepatocyte on Plate **A** while fairly demonstrated on Plate **B,** and short blue arrow: This showing well demonstrated portal triad on Plate **A.**

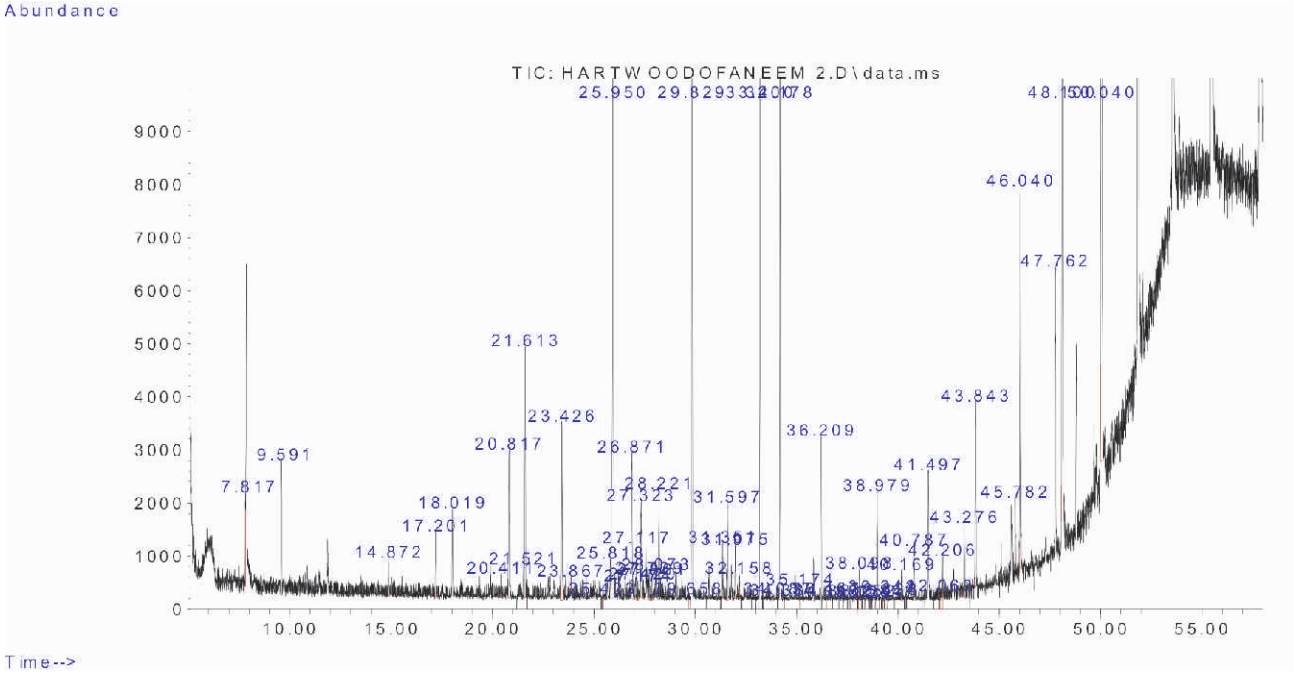

Figure 2: **Chromatogram of GC-MS analysis**

Table 4: Bioactive compounds obtained from the *Azadirachta indica* heartwood extract using GC-MS

S/N	RT	AREA	COMPOUND
1	7.817	0.47 D	(Methylthio)-acetonitrile
2	9.591	1.16 D	1-(2'-Hydroxy-5'-methylphenyl)-1-propanone (E)-oxime
3	14.872	0.37 D	cis-4-Ethoxy-b-methyl-b-nitrostyrene
4	18.019	0.80 D	2-formyl-Cyclopropanecarboxamide, N-benzoyl oxy-
5	20.411	0.05 D	4-(4-methylphenyl)-5-phenoxy-6-phenyl-
6	20.411	0.05 D	4-(4-methylphenyl)-5-phenoxy-6-phenyl-
	20.817	1.55 D	1-Iodo-2-methylnonane
7	21.521	0.14 D	2,3-bis (phenyl amino)-3-(4-nitrophenyl)-1-phenyl-
8	23.867	0.08 D	
9	25.423	0.51 D	3-Phenyl-N-(2'-acetylphenyl)-
10	27.117	0.10 D	3-Ethyl-3-methylheptane
11	27.174	0.99 D	(4-chloro-2-methylphenoxy)-6-ethyloct-3-yl ethyl ester
12	27.323	0.99 D	4-(4-methylphenyl)-5-phenoxy-6-phenyl-2-butenedioic acid
13	27.420	0.04 D	trimethyl-Oxazole
14	27.729	0.15 D	(4-chloro-2-methylphenoxy)-5-phenyl-3-Amino-4,5-dihydro-1-phenylpyrazole
15	27.861	0.13 D	
16	28.073	0.19 D	3-phenyl- N-(2'-acetylphenyl)-2-Ethyl-5-(4-nitro-1,8-naphthalimido)-1,3,4-thiadiazole
17	29.658	0.05 D	
18	31.351	0.34 D	3-Phenyl-N-(2'-acetylphenyl)-7-methanocyclopenta [8]

19	34.087	0.08 D	annulene-3,6-diol
20	34.178	5.04 D	5-(1-naphthalenyl)-1H-Indole-2-carboxamide
21	34.384	0.06 D	Perhydro-htx-2-one 1-Phosphacyclopent-2-ene
22	36.209	1.65 D	
23	38.584	0.06 D	

Discussion

The hazardous effects of synthetic dyes to the environment have led to a reverse approach by researchers to search and use local natural dyes to reduce dependence and limit the use of synthetic dyes. In this study, *Azadirachta indica* heartwood extract solution was used as cytoplasmic stains at different manipulation. Findings in this work revealed comparable and satisfactory demonstration of selected organs by the Haematoxylin and *Azadirachta indica* heartwood extract with Haematoxylin and Eosin method. In this study, it was observed that the *Azadirachta indica* heartwood extract powder dissolved in alcohol well. *Azadirachta indica* heartwood extract was prepared to stain the cytoplasmic components as eosin substitute with satisfactory results. In this study, it was also understood that *Azadirachta indica* heartwood extract could replace eosin in H&E technique. In this study, there was poor staining ability by *Azadirachta indica* heartwood extract solution at 2% compared to haematoxylin and eosin method. The pH of the extracts was found to be acidic, and in this work the pH was modified using 0.5ml of ammonium hydroxide and glacial acetic acid to obtain a suitable pH that will stain the respective tissues, the result obtained in this research still indicated that the extracts are in acidic state which might be due to high acidity of *Azadirachta indica* heartwood extract which is corresponds to eosin in H and E technique In this study, different concentrations (0.5%, 1%, and 2%) under different pH (4-6) and time were used. The result obtained for selected organs shows that 0.5% and 1% *Azadirachta indica* heartwood extract solution showed better staining effect on selected organs which technically signifies that 0.5% and 1% concentration have appropriate staining ability than 2% concentration, which may be due to the method of extraction and modification.

From the study conducted on 0.5%, 1% and 2% of the alcoholic extract of fresh *Azadirachta indica* heartwood extract used as counter stain at pH 5, 4, and 6. And the results of the three different concentration of alcoholic extract stained the cytoplasm pale pinkish while the control H&E, eosin stained the cytoplasm pink-red. It was observed that *Azadirachta indica* heartwood extract solution without mordanting stained the tissue better and the alcoholic extracts improve the binding of the dye, i.e. mediate a dye-tissue interaction. These findings are similar to the report of where different leaf extract from different plant was used as a counterstain (Chukwu, 2011). The results of the study showed that the alcoholic extract of *Azadirachta indica* heartwood extract pH 5, 4, and 6 have affinity to stain cytoplasmic boundaries of the two analyzed tissue sections. The chemical components of *Azadirachta indica* heartwood extract dye responsible for staining is due to the presence of hydro (H+) polar molecule and this may also responsible for its tendency to stain the cytoplasm (Chukwu, 2011). However, the various phytopigment and phytochemical constituent portray the *Azadirachta indica* heartwood extract plant extract as a successful potential natural dye. This may also determine the pH of the solution. Therefore, from the chemical theory of staining, a cell component such as cytoplasm, which is predominantly basic in reaction, is stained by acidic dyes, while a cell component such as the nucleus which is predominantly acidic in reaction is stained by basic dyes (Avwioro, 2014).Characterization of the extract was conducted using GC-MS to identify the active compounds present in the

extract and the results obtained includes (Methylthio)-acetonitrile, 1-(2'-Hydroxy-5'-methylphenyl)-1-propanone (E)-oxime, cis-4-Ethoxy-b-methyl-b-nitrostyrene , 2-formyl-Cyclopropanecarboxamide, N-benzoyl oxy-, 4-(4-methylphenyl)-5-phenoxy-6-phenyl-, 4-(4-methylphenyl)-5-phenoxy-6-phenyl-, 1-Iodo-2-methylnonane, 2,3-bis (phenyl amino)-, 3-(4-nitrophenyl)-1-phenyl-, 3-Phenyl-N-(2'-acetylphenyl)-, 3-Ethyl-3-methylheptane, (4-chloro-2-methylphenoxy)-, 6-ethyloct-3-yl ethyl ester 4-(4-methylphenyl)-5-phenoxy-6-phenyl-2-butenedioic acid, trimethyl-Oxazole, (4-chloro-2-methylphenoxy)-, 5-phenyl-3-Amino-4,5-dihydro-1-phenylpyrazole, 3-phenyl- N-(2'-acetylphenyl)-, 2-Ethyl-5-(4-nitro-1,8-naphthalimido)-1,3,4-thiadiazole, 3-Phenyl-N-(2'-acetylphenyl)-, 7-methanocyclopenta [8], annulene-3,6-diol, 5-(1-naphthalenyl)- 1H-Indole-2-carboxamide, Perhydro-htx-2-one, 1-Phosphacyclopent-2-ene.

Conclusion

The characterization of the extract revealed that some Chromophores and auxochromes containing active compounds were present in the extract. While the staining characteristics of alcoholic extract (0.5% and 1%) of *Azadirachta indica* heartwood at pH 5 and 6 have been established in this study, it is worth noting that alcoholic extract (0.5% and 1%) of *Azadirachta indica* have the closest morphologic resemblance and may be substituted for eosin in histological staining.

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