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Evaluation of *Citrus aurantifolia* (Lime) Juice as A Low-Cost, Locally Available Alternative to Formalin in Histological Fixation of Soft Tissues

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Abstract

This study evaluated freshly prepared *Citrus aurantifolia* (lime) juice as a fixative for Wistar rat tissues compared to 10% neutral buffered formalin (NBF). Liver, kidney, lung, heart, testis, and skeletal muscle tissues of Wistar rats were fixed in lime juice and formalin and compared macroscopically and microscopically. The tissues fixed in lime juice showed no architectural distortion and degeneration compared to tissues fixed in formalin during the first 7 days. From day 14 onward, the liver, kidney, and lung showed progressive microscopic and macroscopic degeneration and pigment deposition. Macroscopically, the tissues fixed in lime juice remained odorless but darkened with precipitate formation over time. Lime juice is an effective short-term fixative for routine histological studies.

***Keywords*:** *Lime Juice, Citrus aurantifolia, Tissue fixation, Histology, Wistar rat*

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I. INTRODUCTION

Histopathological studies rely on tissue fixation for the preservation of cellular architecture for diagnostic examination. According to Carson and Cappellano (2015), and Suvarna *et al*. (2019), the use of 10% natural buffered formalin (NBF) has been the gold histological fixative due to its excellent preservation of nuclear details and its compatibility with routine histological staining protocols. Formalin, however, is dangerous. The International Agency for Research on Cancer (IARC) classifies it as a group human carcinogen, which is the highest risk category. Studies have consistently linked its exposure to nasopharyngeal cancer and leukamia (International Agency for Research on Cancer (IARC, 2012; Zhang *et al*., 2009). Personnels who work in histopathology, anatomy, and mortuary laboratories are exposed to formalin every day. In low-income and middle-income settings, this risk is compounded; ventilation is poor, and protective equipment is scarce and substandard (Igharo *et al*., 2014; Ladeira *et al*., 2011). But formalin causes problems beyond health risks. Its cross-linking action breaks up nucleic acids and hides protein epitopes. This makes tests like PCR, next-generation sequencing, or immunohistochemistry much harder to run reliably (Do and Dobrovic, 2015; Shi *et al*., 2001; Masuda *et al*., 1999).

The search for a safer and sustainable alternative has been triggered because of these challenges. Research on formalin alternatives such as honey and jaggery have shown good preservation of nuclear morphology. However, they have consistently shown poor cytoplasmic detail and suffer from batch-to-batch variability, thereby limiting their diagnostic reliability (Yee *et al*., 2023; Sinha *et al*., 2017; Bhattacharyya *et al*., 2018). Lime (*Citrus aurantifolia*) juice, which is rich in citric acid (about 6 to 8%), antimicrobial phytochemicals, with a low pH (of approximately 2.5), presents a more attracting alternative (Adebayo-Tayo *et al*., 2016; Bamise and Oziegbe, 2013). Its ability to coagulate protein (fixation mechanism) may halt autolysis without toxic cross-linking (Kiernan, 2015). Additionally, lime juice is safe and cheap to get. It is non-carcinogenic, biodegradable, and very easy to get in tropical regions where formalin risks are highest. Despite these benefits and properties, no peer-reviewed study has tested it as a primary fixative. In this study, we decided to test it. The study evaluates lime juice efficacy against formalin in Wistar rat tissues.

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II. Materials And Methods

A. Study design and ethical approval

This study evaluated the fixative efficacy of freshly prepared *Citrus aurantifolia* (lime) juice, its low-cost, and locally available potential in comparison with 10% neutral buffered formalin (NBF). Ethical approval was obtained from the University of Port Harcourt Research Ethics Committee (UPREC), with reference number: UPH/CEREMAD/REC/MM116/033. Our study followed Helsinki 1995 declaration, revised Edinburgh 2000, and national animal research guidelines. To minimize suffering, we adhered to the 3Rs principles: Replacement, Reduction, and Refinement (National Research Council, 2011; Russell and Burch, 1959).

B. Experimental Animals and Tissue Harvest

Five healthy male Wistar rats weighting about 180-220g were obtained from the animal house of the Faculty of Basic Medical Sciences, University of Port Harcourt. They were acclimatized for two weeks under controlled conditions: temperature at 25 ± 2°C, lights on/off every 12 hours, and ad libitum access to commercial pellets and water (Hau and Schapiro, 2021). Each of the animals was anaesthetized using chloroform inhalation. The animals were placed in a sealed chamber with cotton soaked with chloroform until they lost consciousness (American Veterinary Medical Association [AVMA], 2020). The liver, testis, heart, skeletal muscle, kidney, heart, and lung were harvested, rinsed in normal saline to remove blood, and cut into tissue blocks of approximately 3-5mm.

C. Fixative Preparation and Fixation Protocol

We harvested fresh, ripe limes by hand in Amapu, Egbeda, Emohua Local Government Area, Rivers State, Nigeria. The selection criteria included uniform green to yellow-green rinds and firm texture. For botanical verification, samples were taken to the herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt. A qualified taxonomist confirmed the species, and the specimen was archived there for future reference.

The lime fruits were washed thoroughly under running tap water. After which they were cut with sterile knife and were manually juiced into a clean beaker by hand squeezing. The juice was filtered using a sterile muslin cloth into clean labelled sample bottles to remove pulp and seeds. Approximately 350mL of the lime juice was extracted and used immediately, and 30mL was reserved for phytochemical analysis and pH evaluation.

The tissues were assigned to two groups: the experimental group (fresh lime juice) and control group (10% neutral buffered formalin). The tissue-to-fixative ratio was 1:10 (w/v). Sealed bottles were stored at room temperature. Organs were collected for histological processing on day 3, day 7, day 14, day 21, and day 28. The macroscopic features of the organs (colour, texture, odour) were noted at every interval.

D. Phytochemical Analysis and pH Evaluation

Phytochemical screening was carried out to determine the presence of bioactive compounds in the sample. Tests were performed for alkaloids (Dragendorff, Wagner, Mayers, and Hagers reagents), carbohydrates (Molisch and Fehling solution), flavonoids (NaOH, $AlCl_3$, and Shinoda test), anthraquinones, saponins (foaming and emulsion tests), phenols (Ferric chloride), glycosides (Salkowski and Lieberman), cyanogenic glycosides (sodium picrate paper), and phlobatannins (Sofowora, 2008). All analyses were performed in accordance with established standard procedures.

The pH of the sample was determined using a properly calibrated pH meter. Calibration of the instrument was done with buffer solutions of pH 4.0 and 7.0 prior to measurement. The electrode was then inserted directly into the juice sample, and the pH value was recorded once the reading became stable at room temperature.

E. Histological Processing and Staining

The tissues were processed using the conventional paraffin embedding technique at Cedar Pathological and Forensic Services Laboratory, Igwuruta, Port Harcourt. Dehydration was performed through graded ethanol solutions (50%, 70%, 95%, absolute alcohol) for 2 hours per change, followed by three changes of absolute ethanol (6 hours each). Clearing was performed in xylene (three changes, 1 hour each). Infiltration was carried out in molten paraffin wax (melting point: 56–60°C) for two changes (1 hour each). Tissues were oriented in embedding moulds and cooled on a cold plate. Serial sections of 5 µm thickness were cut using a rotary microtome and mounted on glass slides. Sections were dried overnight on a slide-warming tray at approximately 40°C.

Sections were stained with Hematoxylin and Eosin (H&E). Deparaffinization was performed in two changes of xylene (5 minutes each). Rehydration occurred through descending graded ethanol (100%, 95%, 70%, 50%). Sections were stained with Harris's hematoxylin for 5 minutes, rinsed in tap water, differentiated in 1% acid alcohol, and blued in Scott's tap water substitute. Counterstaining with eosin was carried out for approximately 2 minutes. Thereafter, the sections were passed through graded alcohol solutions for dehydration, followed by clearing in xylene (two changes of 5 minutes each). The sections were then mounted using DPX mounting medium. Prepared slides were left to dry in a horizontal position before being examined under the microscope.

F. Microscopic Examination and Data Analysis

The stained sections were examined under a light microscope at magnifications of x100 and x400. Photomicrographs were obtained using a digital imaging attachment (Labomed, Lx400, California, USA). The assessment of tissue preservation focused on nuclear details, cytoplasmic clarity, staining quality, and the overall tissues architecture. To reduce observer bias, the evaluation was carried out by an experienced histopathologist who was blinded to the sample groups. Data analysis was qualitative in nature. Microscopic

features of tissues fixed in lime juice were compared with those fixed in formalin across the different time intervals.

III. RESULTS

A. Phytochemical and pH Analysis

The result of the phytochemical analysis of the lime juice sample shows the presence of carbohydrates, alkaloids (Dagendorff, Wagner, Hagers positive), flavonoids, phenols, steroids/triterpenoids, and glycosides as shown in table 1 below. Saponins, anthraquinones, cyanogenic glycosides, and fixed oils were not detected in the analysis as shown in table 1. The result of the pH analysis shows that the pH of the lime juice sample was 2.45. The table below shows the results of the phytochemical analysis of *Citrus aurantifolia*.

Table 1. Results of the Phytochemical Analysis of Freshly Prepared *Citrus aurantifolia* (Lime) Juice

S/N	ELEMENT	CHARGE
1	Carbohydrate test Fehling solution Molisch	 +ve +ve
2	Alkaloids test Dragendorff Mayers Wagner Hagers	 +ve -ve +ve +ve
3	Flavonoids test Shinoda $AlCl_3$ NaOH	 +ve +ve +ve
4	Saponin test Frothing Emulsion	 -ve -ve
5	Anthraquinone test Free Anthraquinone Combined	 -ve -ve
6	Phenolic test 5% Ferric Chloride	 +ve
7	Steroid/Triterpenoids Salkowski Lieberman-Buchard	 +ve +ve
8	Cyanogenic Glycoside	-ve
9	Fixed Oil	-ve
10	Glycoside test Keller-Killiani Kedde	 +ve +ve
11	Phlobatanin test 1% Hydrochloric Acid	+ve

A. Macroscopic findings

***Days 3-7*:** Macroscopically, the lime juice fixative remained clear and odorless between day 1 to day 7. The lime juice-fixed tissues retained their natural color, soft texture, and elasticity as shown in figure 1A and 1b. Figure 1 (A, B) below shows the appearance of the lime juice and the lime juice-fixed tissues for day 3 and day 7, respectively.

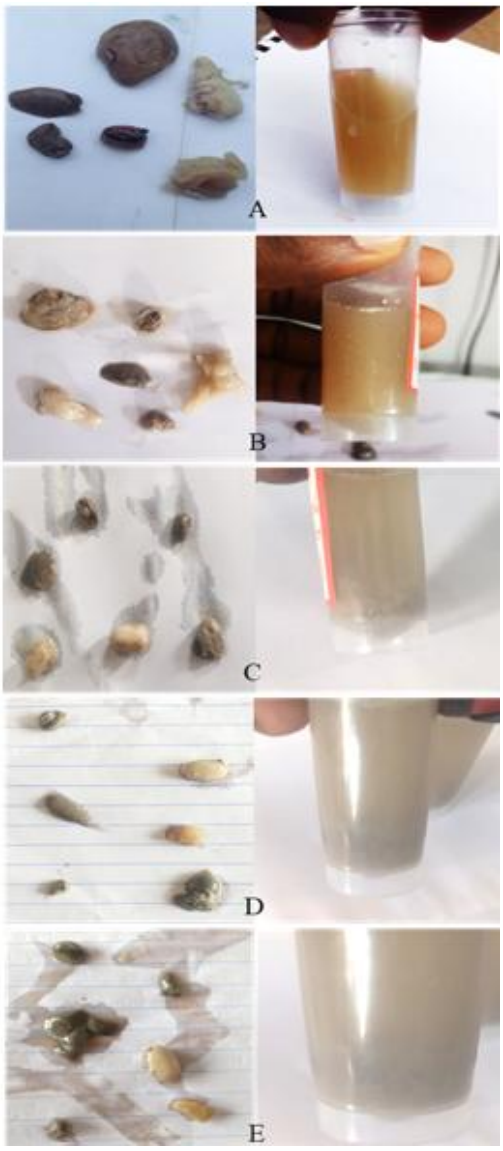

Figure 1. Macroscopic appearance of lime juice and the lime juice-fixed tissues. A, day 3. B, day 7. C, day 14. D, day 21. E, day 28.

Days 14-28: From day 14 onward, there was observable progressive darkening of the lime juice as shown in figure 1C, 1D, and 1E. The fluid developed precipitates and formed a scum layer on the surface of the fluid. Comparably formalin remained intact. Concurrently, the liver, kidney, and lung fixed in lime juice became increasingly dark and soft. It was also noted that the heart fixed in lime juice became decolored while maintaining its firmness. The testis and skeletal muscle maintained original color and firmness throughout with no detectable odor at any stage. Figure 1 (C, D, E) shows the appearance of the lime juice and the lime juice-fixed tissues for day 14, day 21, and day 28, respectively.

B. Microscopic findings

DAY ***3 AND 7***: Liver, kidney, and lung tissues fixed in lime juice showed excellent preservation comparable to formalin: sharp nuclei, distinct cytoplasmic boundaries, intact architecture, no autolysis, as shown in figure 2A to 7B.

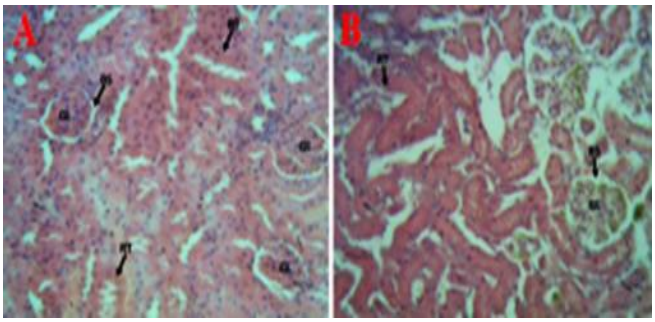

Figure 2. **A**, Photomicrograph of the kidney fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). **B**, Photomicrograph of the kidney fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). (GL = Glomerulus, BS = Bowman's space, RT = Renal tubule). Remark: Tissues well fixed in formalin and lime juice at day 3.

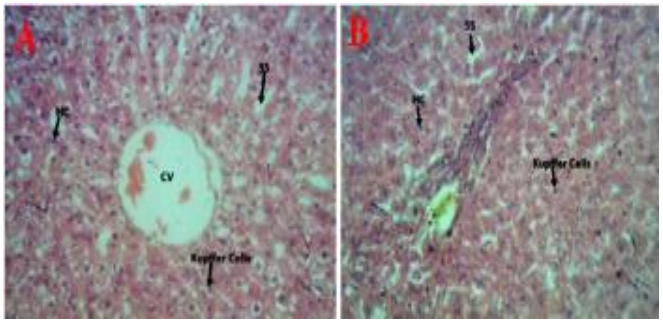

Figure 3. **A**, Photomicrograph of the liver fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). **B**, Photomicrograph of the liver fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). (HC = Hepatocytes, SS = Sinusoids, CV = Central vein). Remark: Tissues well fixed in formalin and lime juice at day 3.

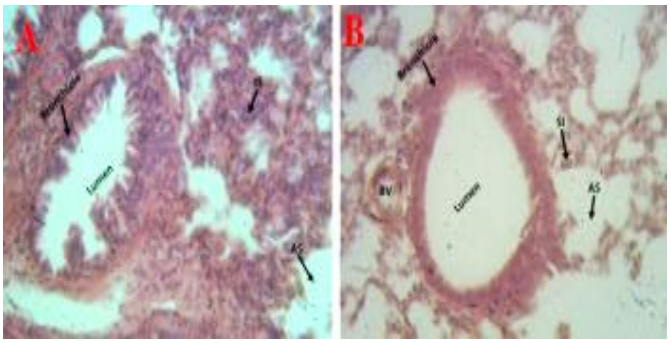

Figure 4. **A**, Photomicrograph of the lung fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). **B**, Photomicrograph of the lung fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 3 (H&E, ×400). (AS = Air sac, IS = Intra-alveolar septa). Remark: Tissues well fixed in formalin and lime juice at day 3.

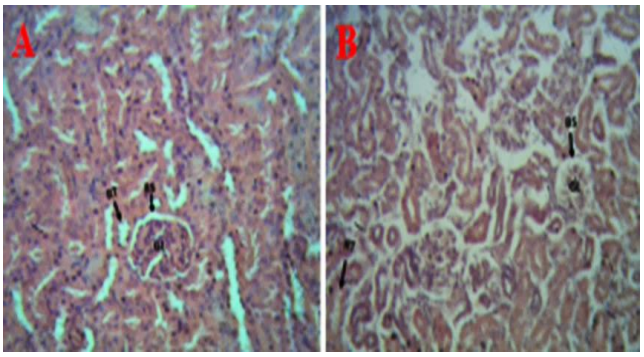

Figure 5. **A**, Photomicrograph of the kidney fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). **B**, Photomicrograph of the kidney fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). (GL = Glomerulus, BS = Bowman's space, RT = Renal tubule). Remark: Tissues well fixed in formalin and lime juice at day 7.

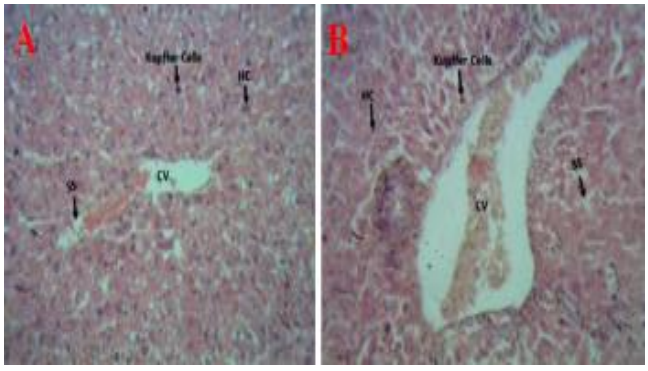

Figure 6. **A**, Photomicrograph of the liver fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). **B**, Photomicrograph of the liver fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). (HC = Hepatocytes, SS = Sinusoids, CV = Central vein). Remark: Tissues well fixed in formalin and lime juice at day 7.

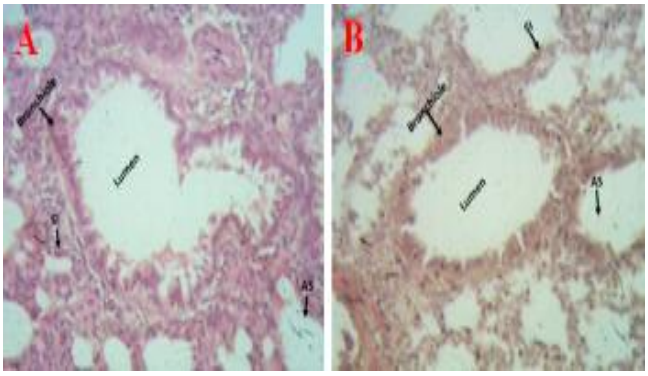

Figure 7. **A** - Photomicrograph of the lung fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). **B** - Photomicrograph of the lung fixed in lime juice, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 7 (H&E, ×400). (AS = Air sac, IS = Intra-alveolar septa). Remark: Tissues well fixed in formalin and lime juice at day 7.

DAY 14: Liver, kidney, and lung exhibited mild architectural distortion and faint yellowish-brown (lemon pigment) deposition, especially for kidney and liver as shown in figure 8B and 9B; however, nuclear detail remained sufficient for evaluation.

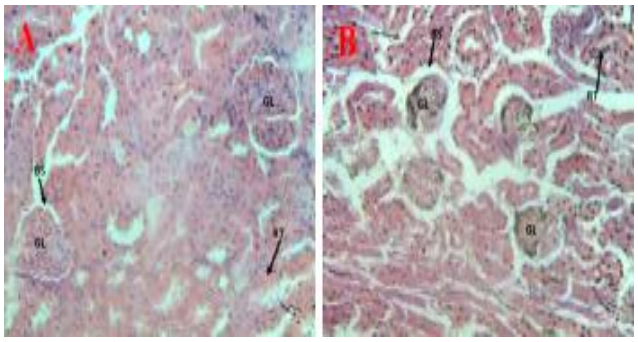

Figure 8. **A**, Photomicrograph of the kidney fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 14 (H&E, ×400). **B**, Photomicrograph of the kidney fixed in lime juice, showing slowly distorting tissue architecture and lemon-brownish coloration at day 14 (H&E, ×400). (GL = Glomerulus, BS = Bowman's space, RT = Renal tubule). Remark: Kidney tissue well fixed in formalin but poorly fixed in lime juice at day 14.

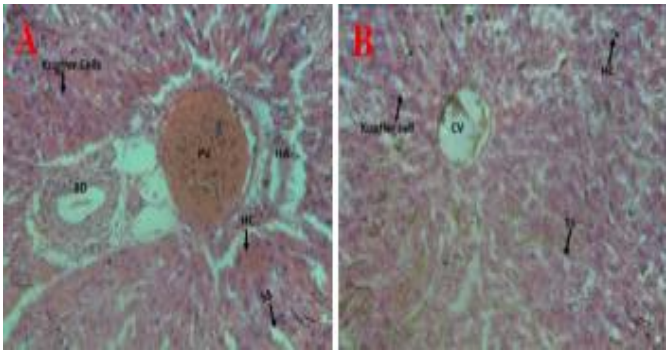

Figure 9. **A**, Photomicrograph of the liver fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 14 (H&E, ×400). **B**, Photomicrograph of the liver fixed in lime juice, showing slowly distorting tissue architecture and lemon-brownish coloration at day 14 (H&E, ×400). (HC = Hepatocytes, SS = Sinusoids, CV = Central vein). Remark: Liver tissue well fixed in formalin but poorly fixed in lime juice at day 14.

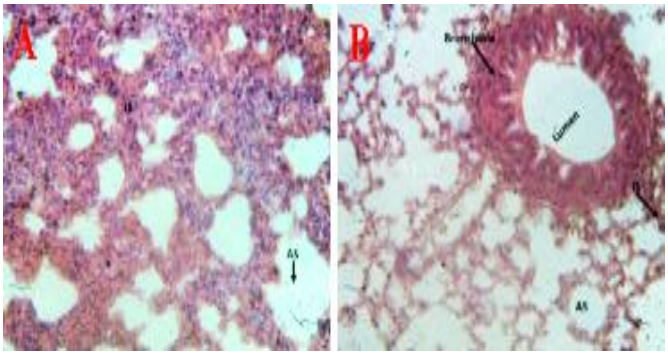

Figure 10. **A**, Photomicrograph of the lung fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 14 (H&E, ×400). **B**, Photomicrograph of the lung fixed in lime juice, showing mildly preserved but distorted alveolar walls at day 14 (H&E, ×400). (AS = Air sac, IS = Intra-alveolar septa). Remark: Lung tissue is well fixed in formalin but mildly fixed in lime juice at day 14.

***DAY 21 AND 28*:** Liver, kidney, and lung tissues fixed in lime juice showed poor preservation: cellular degeneration, loss of structural integrity, nuclear pyknosis, and prominent brown pigment deposits compromising diagnostic clarity, as shown in figure 11B, 12B, 13B, 14B, 15B, and 16B.

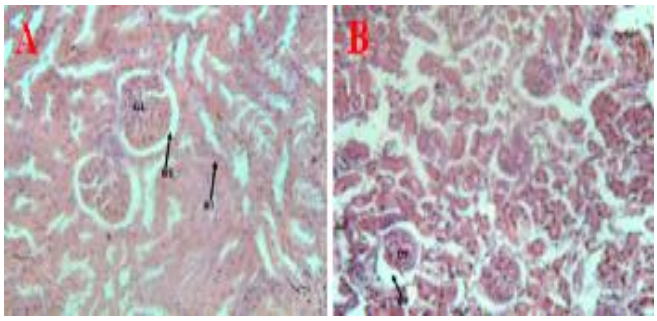

Figure 11. A, Photomicrograph of the kidney fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 21 (H&E, ×400). B, Photomicrograph of the kidney fixed in lime juice, showing distorted tissue architecture at day 21 (H&E, ×400). (GL = Glomerulus, BS = Bowman's space, RT = Renal tubule). Remark: Kidney tissue is well fixed in formalin but poorly fixed in lime juice at day 21.

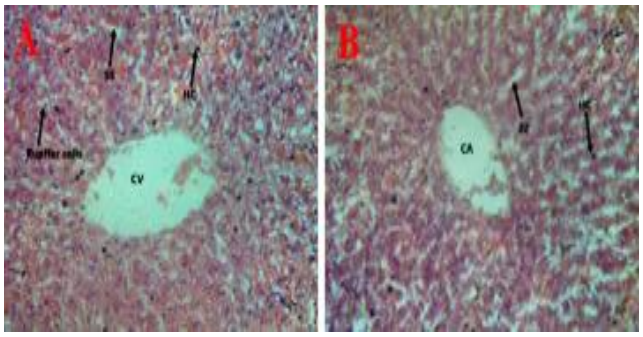

Figure 12. **A**, Photomicrograph of the liver fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 21 (H&E, ×400). **B**, Photomicrograph of the liver fixed in lime juice, showing distorted tissue architecture and lemon-brownish coloration at day 21 (H&E, ×400). (HC = Hepatocytes, SS = Sinusoids, CV = Central vein). Remark: Liver tissue well fixed in formalin but poorly fixed in lime juice at day 21.

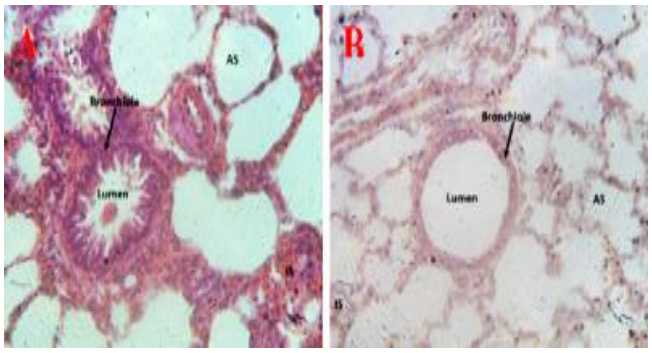

Figure 13. **A**, Photomicrograph of the lung fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 21 (H&E, ×400). **B**, Photomicrograph of the lung fixed in lime juice, showing distorted tissue architecture at day 21 (H&E, ×400). (AS = Air sac, IS = Intra-alveolar septa). Remark: Lung tissue is well fixed in formalin but poorly fixed in lime juice at day 21.

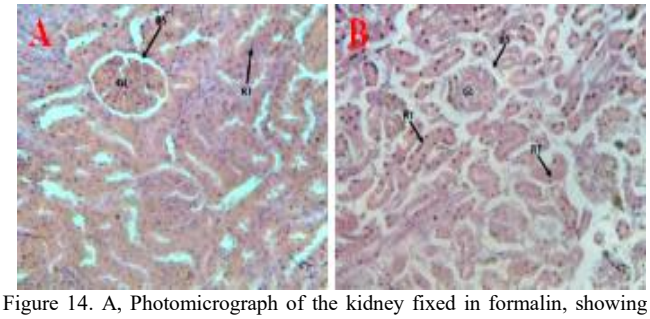

Figure 14. A, Photomicrograph of the kidney fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture

at day 28 (H&E, ×400). B, Photomicrograph of the kidney fixed in lime juice, showing severe distortion of the tissue architecture at day 28 (H&E, ×400). (GL = Glomerulus, BS = Bowman's space, RT = Renal tubule). Remark: Kidney tissue well fixed in formalin but poorly fixed in lime juice at day 28.

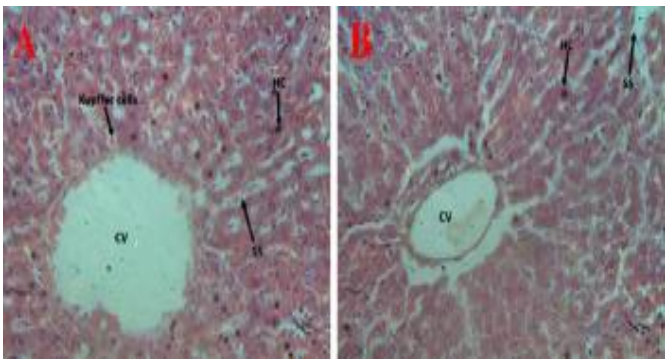

Figure 15. **A**, Photomicrograph of the liver fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 28 (H&E, ×400). **B**, Photomicrograph of the liver fixed in lime juice, showing severe distortion of the tissue architecture and lemon-brownish coloration at day 28 (H&E, ×400). (HC = Hepatocytes, SS = Sinusoids, CV = Central vein). Remark: Liver tissue well fixed in formalin but poorly fixed in lime juice at day 28.

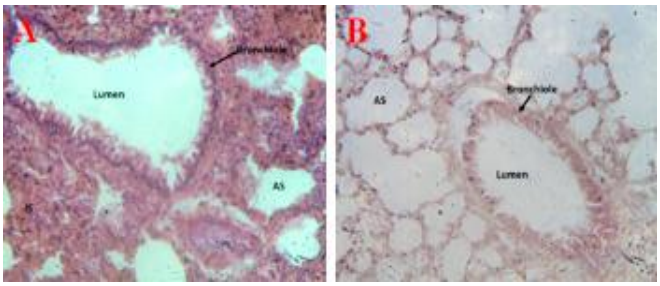

Figure 16. **A**, Photomicrograph of the lung fixed in formalin, showing well-fixed delineated features and appearance of the tissue histoarchitecture at day 28 (H&E, ×400). **B**, Photomicrograph of the lung fixed in lime juice, showing severely distorted tissue architecture at day 28 (H&E, ×400). (AS = Air sac, IS = Intra-alveolar septa). Remark: Lung tissue well fixed in formalin but poorly fixed in lime juice at day 28.

II. Discussion

The results demonstrate that freshly prepared lime juice functions as an effective short-term fixative for mammalian tissues, with observable histological quality comparable to 10% NBF during the first 7 days. This short-term fixative efficacy is primarily as a result of the juice's low pH acidic environment (pH value of 2.45) and its constituents of bioactive phytochemicals; flavonoids, phenols, alkaloids, glycosides, and phlobatanins as shown in table 1.

Studies document that phenolic compounds and flavonoids demonstrate potent antimicrobial and antioxidant activity. The compounds disrupt the cell membranes of microbes, chelate essential metal ions, and inhibit vital enzymatic actions that collectively halt putrefaction (Adebayo-Tayo *et al*., 2016; Cowan, 1999). Alkaloids and phlobatanins also contribute to this preservative effect. They work by intercalating with microbial nucleic acids and precipitating microbial proteins, respectively (Cowan, 1999; Sofowora, 2008). In addition, the highly acidic pH of 2.45, mainly from citric acid, denatures bacterial protein, and creates an environment that suppresses common putrefaction organisms like Clostridium and Proteus species (Adebayo-Tayo *et al*., 2016; Fisher and Phillips, 2008).

One of the notable macroscopic findings was the absence of pungent and foul odor throughout the period of observation (28 days), despite the progressive deterioration of the tissues from day 14. During tissue decay, odor is generally associated with volatile organic compounds (VOCs) like putrescine, cadaverine, hydrogen sulfide, and ammonia. They are bye-products of the putrefaction and proteolysis of bacteria. The low pH and the bioactive antimicrobial activity of the lime juice phytochemicals suppressed and hindered the metabolic activity of the putrefying bacteria. While the endogenous enzymatic autolysis (self-digestion) of the tissues eventually caused the loss of the cellular integrity of the tissues from day 14, the inhibition of bacterial putrefaction suggests that odor-producing volatile organic compounds (VOCs) were never generate. In addition, the acidic environment possibly trapped potential volatile amines in their non-volatile and protonated forms, thereby preventing their release into the surrounding air (Magnaghi *et al*., 2021).

The maintained stability of 10% natural buffered formalin over lime juice is traceable to its robust mechanism of action. The active component of formalin, formaldehyde forms methylene bridges (-CH2-) between the primary amine groups of neighboring proteins. This creates a strong three-dimensional cross-linked network that resists autolysis, putrefaction, and physical degradation of tissue architecture over months or years (Carson and Cappellano, 2015; Suvarna *et al*., 2019; Fox *et al*., 1985). In contrast, lime juice works differently. It relies on coagulative fixation through acid-induced protein coagulation and phytochemical interactions. But these bonds are reversible. They are also susceptible to hydrolysis and ongoing enzymatic degradation over time. This chemical difference explains what we saw on day 14 (mild architectural distortion), and on day 21 and day 28 (severe cellular degradation and loss of structural integrity.

Another factor is the drop in tissue preservation on day 14. This could be directly attributed to the time-dependent changes in the chemical composition of lime juice. Studies show that freshly prepared lime juices become chemically and microbiologically unstable over time (Kaul and Saini, 2000). Citric acid is a predominant organic acid in *Citrus aurantifolia* (Sharma *et al*., 2018), while changes in citric acid concentration have been reported during storage of citrus juice (Robertson and Samaniego-Esguerra, 1990). Such changes may have contributed to the gradual reduction in the preservative effectiveness of the lime juice observed in the study. With longer storage, the breakdown of the citric acid raises the pH. This weakens the acidic conditions needed to inhibit microbial growth and tissues autolysis (Burdurlu *et al*., 2006).

Moreover, the identified phytochemical constituents of lime juice may contribute to its antimicrobial activity. Oikeh *et al*. (2016) demonstrated the presence of several phytochemicals, including alkaloids, flavonoids, steroids, terpenoids,

saponins, cardiac glycosides, and reducing sugars in *Citrus aurantifolia* juice and reported antimicrobial activity against bacterial and fungal organisms. Some bioactive constituents of citrus juices are susceptible to degradation during storage. In particular, vitamin C degradation in fresh citrus juices, including lime juice, follows first-order kinetics and is influenced by storage time and oxygen exposure (Nakilcioglu-Tas and Otles, 2020). Similar first-order degradation of vitamin C has also been reported during storage of other citrus juice concentrates (Burdurlu *et al*., 2006). In *Citrus aurantifolia,* storage has likewise been associated with reductions in several juice constituents, including ascorbic acid and hesperidin (Kaul and Saini, 2000). These time-dependent compositional changes may contribute to the gradual reduction in the antimicrobial and preservative effectiveness of the juice observed in the present study. This chemical instability possibly accounts for the macroscopic changes observed; darkening, precipitate formation, and scum layer development in lime juice from day 14 of the fixation period onward.

Microscopically, the yellowish-brown pigment that appeared at day 14 and became very prominent at day 21 and day 28 is a critical factor that limits lime juice's archival utility. The discoloration resembles that of jaggery (unrefined sugar cane) fixation. Microscopically, the yellowish-brown pigment that appeared at day 14 and became very prominent at day 21 and day 28 is a critical factor that limits lime juice's archival utility. The discoloration resembles that reported in jaggery-fixed tissues. Sinha *et al*. (2017) and Joy *et al*. (2024) reported a brownish tint in jaggery-fixed specimens. Similarly, pigment deposition in lime-fixed tissues likely results from acid-induced oxidation and polymerization of phenolic compounds and flavonoids present in the juice, or from precipitation of denatured cellular proteins. While lime juice and jaggery offer safe, cost-effective, and locally available alternatives to formalin, both share this limitation; pigmentary artifacts develop over extended time.

Notwithstanding, despite the long-term architectural degradation and pigment deposition, the safety, cost-effectiveness, and local availability of lime juice present a compelling advantage in resource-limited settings. In environments where the procurement, storage, and safe disposal of formalin are challenging, and where occupational exposure poses significant health hazards (Igharo *et al*., 2014; Ladeira *et al*., 2011), lime juice serves as a highly viable alternative for short-turnaround diagnostic settings, intraoperative consultations, or educational histology where tissues are processed within the first 7 days.

A. Study limitations and Future Directions

The limitations of the study are as follows; the assessment employed purely qualitative analysis without a robust standardized scoring, limited to only H&E staining evaluation, the compatibility of lime juice-fixed tissues with immunohistochemistry or molecular techniques remain unknown, only healthy rodent tissues were used, only soft tissues of Wistar rats were used – no hard tissue was used

Further research should do the following: (1) employ standard quantitative analysis, scoring and statistical validation, (2) test the compatibility of the lime juice-fixed tissues with special stains and molecular assays, (3) validate the findings of this study on human specimens and disease tissues of Wistar rats (4) modify the formulation of lime juice to enhance long-term stability, and (5) pilot lime juice in field-based settings where formalin logistics are constrained.

II. Conclusion

Freshly prepared *Citrus aurantifolia* (Lime) Juice is an effective short-term fixative for routine histological studies of Wistar rat soft tissues. Its safety profile, low-cost, and local availability make it a promising candidate for further study, particularly in settings where resources are scarce.

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