UNIVERSITI PUTRA MALAYSIA

EFFECT OF IMMERSION FIXATIVES ON HISTOLOGICAL FEATURES OF RED TILAPIA (*OREOCHROMIS SPP*.) GILL, SKIN AND MUSCLE TISSUES.

NOR AMIRAH BT MOHAMAD ZAMRI

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FPV 2020 77

EFFECT OF IMMERSION FIXATIVES ON HISTOLOGICAL FEATURES OF RED TILAPIA (*OREOCHROMIS SPP*.) GILL, SKIN AND MUSCLE TISSUES.

NOR AMIRAH BT MOHAMAD ZAMRI

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM)

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

2020/2021

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CERTIFICATION

It is hereby certified that we have read this project entitled "Effect of immersion fixatives on histological features of Red Tilapia (*Oreochromis spp*.) gill, skin and muscle tissues", by Nor Amirah bt Mohamad Zamri and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project.

____

ASSOCIATE PROFESSOR DR INTAN SHAMEHA ABDUL RAZAK

DVM (UPM), PhD (UPM)

Lecturer, Faculty of Veterinary Medicine, Universiti Putra Malaysia

(Supervisor)

____

DR MOHD FUAD BIN MATORI

DVM (UPM)

Veterinarian, Aquatic Animal Health Unit, Universiti Putra Malaysia

(Co-Supervisor)

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DR AWANG HAZMI AWANG JUNAIDI

DVM (UPM), PhD (Saskatchewan)

Lecturer, Faculty of Veterinary Medicine, Universiti Putra Malaysia

(Co-Supervisor)

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DEDICATIONS

This project paper is dedicated to

My Beloved Family

My Lecturers

My Friends

&

Putih, Toyen, Tamtam

Thank you for such a wonderful contribution and continuous support.

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ACKNOWLEDGEMENTS

First and foremost, praises and thanks to Allah S.W.T. for His showers of blessings upon me and giving me the strength to complete my final year project successfully.

I would like to express my profound appreciation and gratitude to my supervisor, AP Dr Intan Shameha Abdul Razak for guiding and supervising me dearly with her full dedication, motivation patience and enthusiasm. My deepest gratitude, appreciation and thanks to Dr. Mohd Fuad Matori and Dr. Awang Hazmi bin Awang Junaidi, as my co-supervisors, for their excellent experience and technical guidance.

I extend my thanks and appreciation to the staff of the Veterinary Histology Laboratory, En Nor Hasmadi, Veterinary Histopathology Laboratory, Pn Jamilah, Pn. Latifah, Cik Aisyah and Animal Aquatic Health Unit of the Faculty of Veterinary Medicine, En Azmi and En Zainal whom has assisted and guided me in performing this project.

My deepest love and guidance towards my parents, Madam Norhayati binti Abd Khadir, my siblings, Mohd Amir Ridzuan bin Mohd Zamri and Mohamad Arif Ashraf bin Mohamad Zamri and to rest of my family members.

Last but not least, I would like to further express my gratitude to all my friends, FYP-mates and course mates DVM2021 and everyone who have contributed directly or indirectly in completing this project. Thank you and may everyone be well and healthy always.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Tahun Akhir.

KESAN FIKSATIF SECARA RENDAMAN TERHADAP CIRI-CIRI HISTOLOGI INSANG, KULIT DAN TISU OTOT TILAPIA MERAH (*Oreochromis spp.*).

OLEH

NOR AMIRAH BT MOHAMAD ZAMRI

2020

Penyelia: Prof. Madya Dr. Intan Shameha binti Abdul Razak

Penyelia bersama: Dr Mohd Fuad bin Matori

Dr. Awang Hazmi bin Awang Junaidi

Pada masa kini, minat terhadap penyelidikan histopatologi ikan semakin meningkat. Kaedah fiksatif merupakan salah satu langkah penting dalam penyelidikan ini. Fiksatif yang ideal adalah fiksatif yang dapat memelihara morfologi seakan dengan keadaan asal. Rawatan penyahkalsifikasi juga diperlukan untuk menghilangkan kalsium fosfat dari tisu yang dikalsifikasi sehingga menjadikan tisu tersebut cukup lembut untuk dipotong oleh mikrotom. Sehingga kini, masih ada kekurangan informasi mengenai penggunaan fiksatif dan penyahkalsifikasi yang sesuai bagi menghasilkan mikrograf histologi yang baik dalam tisu ikan. Oleh itu, tujuan kami adalah untuk menentukan

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fiksatif yang paling sesuai digunakan khusus untuk memelihara struktur insang, kulit dan tisu otot ikan Tilapia Merah (Oreochromis spp.) dan rawatan penyahkalsifikasi yang paling terbaik. Lima kawasan tertentu pada sampel dipilih dan dinilai dalam bentuk skor pemarkahan berdasarkan setiap parameter; kejelasan morfologi nukleus, intensiti pewarnaan, integriti tisu-tisu lain dan tahap kemudahan pemotongan spesimen. Jumlah data pemarkahan dikumpulkan dan analisis statistik antara kumpulan diperiksa dengan menggunakan SPSS (versi 25) dengan ujian Kruskal-Wallis. Melalui keputusan yang didapati, larutan Bouin mempunyai skor total tertinggi untuk semua parameter sampel insang, justeru menjadikan larutan Bouin adalah fiksatif terbaik yang digunakan untuk memperbaiki insang. Sementara itu, untuk tisu kulit dan otot, 20% formalin mempunyai jumlah skor tertinggi yang dikumpulkan justeru menjadikan fiksatif ini sebagai fiksatif terbaik yang digunakan untuk memperbaiki kulit dan juga tisu otot. Bagi rawatan penyahkalsifikasi yang terbaik, kalsifisaki yang paling sesuai digunakan dalam kajian ini adalah asid formik yang digunakan selama 10 jam berdasarkan parameter yang dinilai iaitu tahap kemudahan untuk memotong spesimen. Pada masa hadapan, faktor lain yang mempengaruhi kualiti fiksasi seperti tempoh fiksasi, suhu dan juga pH juga harus dinilai untuk menyempurnakan kajian ini.

Keywords: Tilapia merah (*Oreochromis spp.*), fiksatif, rawatan penyahkalsifikasi, insang, kulit, otot.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999- Final Year Project

EFFECT OF IMMERSION FIXATIVES ON HISTOLOGICAL FEATURES OF RED TILAPIA (OREOCHROMIS SPP.) GILL, SKIN AND MUSCLE TISSUES.

By

NOR AMIRAH BT MOHAMAD ZAMRI

2020

Supervisor: Assoc. Prof. Dr. Intan Shameha binti Abdul Razak

Co-supervisor: Dr Mohd Fuad bin Matori

Dr. Awang Hazmi bin Awang Junaidi

There are increasing interests in histopathological investigation of fishes nowadays. Fixation is one of the important steps in the histology process and an ideal fixative is a fixative that can preserve morphology as close to the living state as possible. A decalcification treatment is crucial to remove calcium phosphate from the calcified tissue to make the tissue soft enough to be sectioning. Up until now, there is still a lack of information about the usage of suitable fixatives and decalcifiers that produce good histological micrograph in tilapia fish tissue. Thus, in this study, we aim to determine the most suitable fixatives and decalcification treatment specifically to fix the gill, skin and muscle tissues of the Red Tilapia (*Oreochromis spp.*). In this study, four types of

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fixatives namely 10% Buffered Formalin, 20% Buffered Formalin, Bouin's solution, Davidson's solution and 2 types of decalcifiers which are formic acid with 10% formalin and formic acid with sodium citrate were used. The abovementioned tissues were collected from five fish and put in the different fixatives before processed accordingly and stained with routine staining for histological observation. Five regions of interests were randomly selected and scored on these parameters; overall morphological clarity of nuclei, staining intensity, the integrity of other tissues, lifting of the cartilage, space formation and the ease to section a specimen. The scoring data was collected and the statistical analysis was done. Based on the results, Bouin's solution has the highest total mean score of all parameters for gills sample, but the 10% Formalin and 20% Formalin solution are also the suitable fixatives that used for gill. Meanwhile, for skin and muscle tissues, 20% formalin recorded the highest total mean score, but the 10% Formalin and Bouin's solution also can be used for skin, while for muscle, Bouin's solution also suitable to use as fixatives. For the best decalcification treatment, the most suitable decalcifiers used is buffered formic acid for 10 hours based on the ease to section a specimen parameter evaluated. In conclusion, different fixatives will yield different results in a different type of tissues. However, other factors should be considered such as duration of fixation, the temperature of the fixative and also its pH for future works.

Keywords: fixatives, decalcification treatment, gill, skin, muscle, Red Tilapia (*Oreochromis spp.*)

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1.0 INTRODUCTION

There are increasing interests in histopathological investigation of fishes nowadays. But the histological works are tedious and need to be meticulously processed. One of the important steps is fixation. The purpose of tissue fixation is to preserve morphology as close to the living state as possible (Hopwood, 1985). Conventional 10% formalin solution has been widely used as the histological fixative for fishes. Formalin solution has the characteristic of rapid penetration into tissues and slowly reacting with tissue proteins especially in the formation of methylene bridges but it can also cause defective preservation of some tissue in intact fish bodies. Therefore, Bouin's and Davidson's solutions, which are rapidly permeable fixatives have been recommended to use for the fixation of fish (Miki et al., 2018).

According to Speilberg et al. (1993), they compared five different fixatives, Bouin's fluid (BF), Davidson's fluid (DF), 10% buffered formalin, 10% formalin + 1% glutaraldehyde, and Karnovsky's fluid, for use in light microscopic examination of the liver morphology in Atlantic salmon and concluded that 10% buffered formalin and Davidson's fluid was most suitable for evaluation with Hematoxylin and Eosin (H&E)-stained sections.

There is a little amount of studies carried out regarding the most suitable fixatives used to preserved tissue sample in fish. Different tissues may respond differently in different fixatives. Thus, it is beneficial to carry out this study to compare between fixatives namely which one is the most suitable to preserved tissue sample of gill, muscle and skin in fish.

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Each gill is supported by a cartilaginous or bony gill arch. Sectioning processed will be difficult if the cartilaginous structure does not soften. In order to soften the cartilage tissue, decalcification treatment is used to decalcify the gills, same goes to the skin of fish. Decalcification of tissues is a routine process carried out in most laboratories by the use of various acids or chelating agents. However, it is an inherently complex process, complicated by the trade-off between the time taken for the process and the quality of the sections produced. The fastest result but the poorest quality of sections provided by the stronger acid, the slowest result but the best sections provided by chelating agents (Prasad et al*.,* 2013). Thus, a study on the best decalcification method need to be carried out to identify the method that combines the highest quality of stain and sections with the optimum time.

Thus, the objectives of the study are:

1. To identify the most suitable fixative to preserve gill, skin and muscle tissues of Red Tilapia (*Oreochromis spp*.).
2. To determine the best decalcification treatment used to decalcify gill and skin tissues of the tilapia fish.

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2.0 LITERATURE REVIEW

2.1 Red Tilapia (*Oreochromis Spp*.)

The main cultivated freshwater species in the world belong to carps and tilapia. The Nile tilapia, *Oreochromis niloticus* is one of the most important freshwater fish in world aquaculture. It is widely cultured in many tropical and subtropical countries. The farmed tilapia production worldwide was over 5.8 million tons annually in 2017 and China is the largest tilapia producer in the world (FAO, 2019). Red tilapia is a name used for several different man-made tilapia variants that have an attractive red colouration of the body.

Tilapias are popular cultured species because of their high environmental tolerant characteristics. According to Ghada (2017), tilapia is well suited for aquaculture due to rapid growth, poor quality resistance, ability to grow under sub-optimal nutritional conditions, and high fecundity. These characteristics make them one of the important fish species which has several good qualities and can face a wide range of salinity and other environmental conditions. They can grow well in water salinities ranging from 11 ppm to 29000 ppm and tolerate temperatures between 8°C to 42°C. Though tilapias normally live in freshwater, some could be raised in brackish water or seawater because they show high salt tolerance (Kamal, 2005).

The commonest technique in rearing Red Tilapia in Indonesia is semi-intensive and intensive systems. The intensive culture has usually a high level of management input such as feed and fertilizers are intensively applied following appropriate recommended rates. Most commercial farm adopts this approach due to the fish grow is faster compared to the others (Maia et al., 2016).

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2.2 Gill of fish

The gill is located in the lateral wall of the mouth cavity, which forms a pathway for the water to flow from the mouth to the exterior, where the tissue later becomes the gill arch and supports the gill filaments (Olson, 2011). The anatomical structure of fish gills comprises a row of several arches, with each arch projecting two filaments with a series of lamellae.

Each of the arches supports paired rows of long blade-like filaments where gaseous exchange occurs (Ferguson, 2006). There are two types of muscles, the adductor and the abductor muscles, connected to these arches as mechanical supports to connect the cartilaginous rod in the filament to the arch (Evans et al., 2005). The contraction of the muscles generates a motion allowing the buccal cavity movements during respiration.

The functional anatomical unit of the gill is the gill filament or primary lamellae, it is an osseous structure covered by epidermal tissue (stratified epithelium) containing several cellular types, such as mucous cell, chloride cell, and pavement cells. It also consists of a centrally placed rod-like central venous sinus. The long, slender and flat gill primary lamellae support numerous respiratory secondary lamellae. The gill primary lamellae offer minimal resistance to the flow of water over it and counter-current mechanism of blood and water flow across the gill filament ensures gaseous exchange (Olson, 2011).

The respiratory lamellae or secondary lamella of the gill filaments are plate-like structures projected at right angles from both sides of the filaments. They spread the blood out into the periphery of the tissue creating a minimal diffusion distance

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between the blood and the water (Ferguson, 2006). Each lamella consists of two rows of epithelial cells held apart by a series of centrally located cells called pillar cells. Pillar cells provide structural support to hold two squamous epithelial cell layers together.

2.3 Skin of fish

The skin of all fish species, like that of any other vertebrate, consists of two basic layers which are an outer (the epidermis) and an inner (the dermis). The outermost layer of the epidermis is lined by stratified squamous epithelium. Various specialized cells, including goblet cells, sensory cells, alarm cells and chloride cells may be present in the epidermis (Whitear, 1986).

Rakers (2009) states that the fish epidermis is separated from the underlying dermis by a layer of filamentous proteins, which form the basement membrane. The dermis is located in between the epidermis and the underlying body musculature. In fish with scales, the superficial layer of the dermis is covered by scales. The scales are arranged in scale pockets made up of loose connecting tissues. The loose connective tissue is also known as stratum spongiosum while the dense connective tissue is known as stratum compactum.

A high number of goblet cells that are well distributed among the epithelium contributing to the secretion of mucous with antimicrobial properties (Mokhtar, 2017). The mucus is produced by goblet cells of the stratum spinosum. Their structure is similar to that of mammalian goblet cells. Mucus goblet cells die when they release their glycoconjugates, hence there is a continuous turn over in the outer layers of the epidermis. Alongside the mucus goblet cells, club cells and sensory cells are

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embedded in this layer. These cells exhibit enormous metabolic reaction capacities to meet various external influences.

2.4 Muscle tissue of fish

Muscles are composed of a collection of muscle fibres, held together by loose connective tissues. The body musculature is fairly simple in the fish. The fins are usually provided with individual small muscles, but the most complex organisation is in the head region. Many individual muscles show an arrangement of fibres into bundles separated from each other by connective tissue portions.

The lateral nerves generally penetrate a muscle units' side and branch out as they penetrate the connective tissue. Segmentation or metameres of vertebrate musculature is seen clearly in the lateral muscles of the fishes. They are divided into myotomes or muscle segments, each of which is bent into a single V with the angle directed anteriorly. Each myofibril is composed of two types of short myofilaments which are precisely arranged giving the appearance of transverse banding, the striations

Doaa (2017) stated that there are two most distinctly different skeletal muscle types which are red muscle and white muscle. Both have a different degree of vascularization and myoglobin content, which account for their colour. The layer of red muscle has high lipid content than the white tissues and a larger number of mitochondria per cell and higher respiratory activity. Meanwhile, the white muscle fibre is characterized by a low number of mitochondria and low respiratory activity.

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2.5 Fixatives

Foundation of all good microscopic preparations depends on the treatment of tissue as soon as it is removed from the body. The tissue should be immediately transferred and fixed in an appropriate fixative solution. The valuable details will be missed out in the specimens if the tissue is unfixed or dried out (Rajanikanth et al., 2015). Fixation is considered as a physiochemical process where cells or tissues are fixed chemically. It performs various functions such as prevention of autolysis and tissue putrefaction.

There is various type of fixative agents include formaldehyde, glutaraldehyde, osmium tetroxide, glyoxal and picric acid. Formalin solution has the characteristic of rapid penetration into tissues and slowly reacting with tissue proteins, but it has two well-known disadvantages which are highly carcinogenic and a poor preserver of nucleic acids (Patil et al., 2015). For more than 20 years, there have been numerous attempts to find a substitute, with as many different alternative fixatives, none successful.

Alternatives of formalin solution, Bouin's solution and Davidson's solution also are commonly used in aquatic research. Miki et al. (2018) stated that Bouin's solution or Davidson's solution is designed to rapidly penetrate tissues by mixing with formalin. However, both fixatives have a defect in that they caused artefactual space formation through the shrinking effect of picric acid and ethanol. They also have another defect in that they cause haemolysis through the lytic effect of acetic acid.

2.6 Decalcification solutions

Decalcification is the process of removing calcium from tissues. Inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissues in order to obtain satisfactory paraffin sections of bone. This decalcification process is carried out by chemical agents, either acid to form soluble calcium salts or chelating agents which bind to calcium ions.

Determination of the choice of decalcification technique entails a balance between speed of decalcification and maintenance of tissue morphology and quality of staining. (Kapila et al., 2015). There are two types of decalcifiers which are acids and chelating agents. Acid decalcifiers can be divided into two groups: strong (inorganic) and weak (organic) acids. Formic is the only weak acid used extensively as a primary decalcifier, either to use it as a simple effective decalcifier (10% formic acid), to buffered with sodium citrate (Evans and Krajian, 1930) or to fix and decalcify at the same time in 40% Formaldehyde (Gooding and Stewart, 1932).

Hydrochloric acid and nitric acid are a type of strong acid that can decalcify rapidly but lead to damage to tissues and cause deleterious changes in tissue morphology and stainability (Prasad and Donoghue, 2013). Thus, formic acid is the preferred decalcifier, which while being relatively slower in action, is less damaging to tissues.

However, acids also affect ability of tissues staining and cause morphological alterations such as oedema, shrinkage, vacuolation and disruption (Fernandes et al., 2007). Other than formic acid, a mixture of picric acid, formalin, and acetic acid in Bouin’s solution makes it as a popular combined fixative/decalcification solution that

acts rapidly and provides excellent histology (Jessica, 2002). However, acetic and picric acids may also cause tissue swelling. Thus, by adding the salts or sodium citrate to formic acid solutions, it will produce 'acidic' buffers (Evans and Krajian, 1930). Buffering is used to counteract the injurious effects of the acid.

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3.0 MATERIALS AND METHODS

3.1 Experimental Fish

This study was approved by the Institutional Animal Care and Use Committee (IACUC, UPM) with the AUP number UPM/IACUC-U044/2020. A total of five Red Tilapia (size of 15 to 20 cm in length) were used in this study. All the Red Tilapia were obtained from a commercial supplier located in Kajang, Selangor.

3.2 Experimental Design

The fish were acclimatized for 1 week in a tank prior to trial, in aerated water with the same water parameter and water quality. The fish were fed with the same feeding regime using the commercial feed. The water supply to the laboratory comes from the main supply which passed through series of the dechlorinating unit and the water was changed every 2 days.

The fish was euthanized by deep anaesthesia using 200 mg/L clove oil using the immersion technique followed by pithing technique. Then, the second-gill arch, skin and muscle were immediately removed from the carcass through dissection. From a total of 5 fish, each organ of each fish was divided into 6 groups which are group A (10% Formalin solution with Decalcification treatment a), group B (10% Formalin solution with Decalcification treatment b), group C (20% Formalin solution with Decalcification treatment a), group D (20% Formalin solution with Decalcification treatment b) group E (Bouin's Solution) and group F (Davidson's Solution). The samples were fixed in the fixatives for 24 hours. Then all the samples were taken from

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the fixatives and processed for histological observation by using the routine histological technique (H&E staining).

3.3 Fixatives and Decalcification

Before euthanizing the fish, the 10% Formalin solution (10%FS), 20% Formalin solution (20%FS), Bouin's solution (BS) and Davidson's solution (DS) were prepared by mixing each reagent listed in Table 1.

Two types of decalcification solution (Table 2) also were prepared to decalcify gill and skin tissue after being fixed in the fixatives.

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Table 1: Composition of each fixative. The volume of each reagent is shown as a total volume of 100 mL for each fixative.

Reagents	**Fixatives**			
	10% Formalin Solution	**20% Formalin Solution**	**Bouin's Solution**	**Davidson Solution**
37% Formaldehyde	10mL	20mL	25mL	22mL
Anhydrous ethanol	-	-	-	33mL
Saturated picric acid	-	-	75mL	-
Glacial acetic acid	-	-	5mL	12mL
Distilled water	90mL	80mL	-	33mL

Table 2: Type of decalcification agent used.

Decalcification treatment			
Decalcification a		Decalcification b	
Formic acid	10mL	Sodium citrate	50g
Distilled water	100mL	Distilled water	250mL
10% Formalin	5mL	88% Formic acid	125mL
		Distilled water	125mL
Leave tissue in for **6 hours**		Leave tissue in for **10 hours**	

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Immediately following euthanasia, from a total of 5 fish, each dissected gill, skin and muscle of each fish were divided into 6. Those 6 pieces of the organ were fixed in 6 groups of fixatives protocol which are group A, B, C, D, E and F.

For Group A and B, the samples were fixed in 10% FS for 6 hours at 4°C followed by another 18 hours at room temperature. After 24 hours, samples of group A were transferred to decalcification solution A for 6 hours meanwhile for group B, the samples were transferred to decalcification solution B for 10 hours. For group C and D, double fixation method was used. The samples were fixed in 20% FS for 4 hours and then transferred into 10% FS solution for another 20 hours. The first 6 hours were at 4°C followed by another 18 hours at room temperature. After 24 hours, group C samples were then transferred to decalcification solution A for 6 hours meanwhile for group B, the samples were transfer to decalcification solution B for 10 hours. For group E, the gills, skin and muscle tissues were fixed in Bouin's solution for 24. The samples were then washed with 70% alcohol for 30 minutes. This action was repeated for 3 times to remove the remnant of picric acid prior to staining. Lastly, for group F, the samples were fixed in Davidson's solution for 24 hours. The samples then transferred into 70% alcohol solution for storage purposes. A diagrammatic diaphragmatic illustration of the steps involves from the immersion of samples into fixatives until obtaining histological slide is shown in Figure 1.1.

3.4 Preparation of histological slides

Processing of gills, skin and muscle were performed using an automated tissue processor, where tissue samples were dehydrated through 100 % alcohol and cleared with several baths of xylene. Then, the tissue samples were infiltrated with paraffin

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wax at 71°C using a Leica EG1160 paraffin-embedded station. The gills, skin and muscle were carefully placed level on the bottom of the cassette by gentle pressing from the blunt end of forceps. After cooling and trimming process, the tissue blocks were carefully trimmed (15 µm) to expose tissue and then 4 µm thick paraffin sections were made using a Leica RM2235 microtome. The sections were dried at 48°C on drying cabinet for a minimum of 12 hours prior to staining. The 5 µm thick paraffin wax sections were then stained with H&E. Mounting of glass slide using Distyrene Plasticizer Xylene (DPX) was done after the staining process and let it dry overnight. Finally, the slides are ready to be viewed.

3.5 Histological and morphological observation

All the slides were viewed under the light microscope equipped with a digital camera (Image Analyzer (Olympus BX51) and the parameters in the sampled gills, skin and muscle were scored accordingly. Each slide was viewed with 5 fields under microscope. Gills, skin and muscle were evaluated for parameters listed in Table 3.

Table 3: Histological parameters for gills, skin and muscle.

	GILLS	SKIN	MUSCLE
1	Intensity of H&E stain	Intensity of H&E stain	Intensity of H&E stain
2	Lifting of the cartilage tissue	Lifting of the epithelium	Space formation between muscle fiber
3	Disorganization of secondary lamellae	Space formation between dermis	Gap formation between myofibrils
4	Space Formation	Ease to section a specimen	Ease to section a specimen
5	Lifting of respiratory epithelium		
6	Rupture of lamellae epithelium		
7	Nuclear morphological clarity		
8	Ease to section a specimen		

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All the parameter except for “ease to section a specimen” were graded based on a scoring system from 1-4 scale where 1 indicate very poor and 4 indicate very excellent. Meanwhile for parameter “ease to section a specimen”, the parameter was graded based on scoring system from 1-3 scale. Both scoring systems was summarised in Table 4.

The average from all 5-field view scoring from by each parameter was taken, recorded and analysed.

Table 4: Histological Evaluation Scoring system. Modified method of Miki *et al*., (2018).

Scoring system for all parameters except for “ease to section a specimen”	
Score	**Descriptions**
1	Very Frequent
2	Frequent
3	Rare
4	Absent
Scoring system for parameter “ease to section a specimen”	
Score	**Descriptions**
1	Easy
2	Slightly difficult
3	Very difficult

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a.

Gills, skin and muscle tissues

b.

20%FS

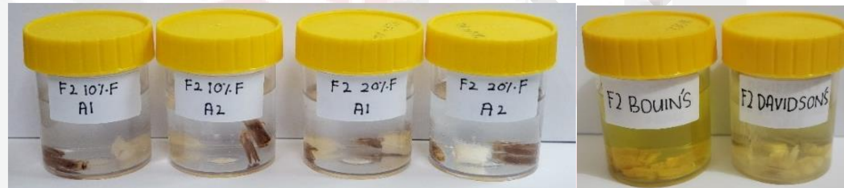
BS

10%F

DS

c.

d.



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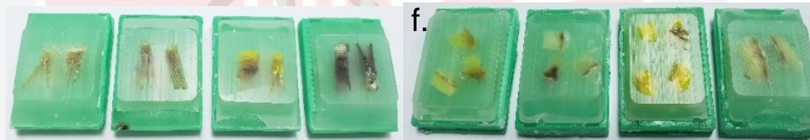

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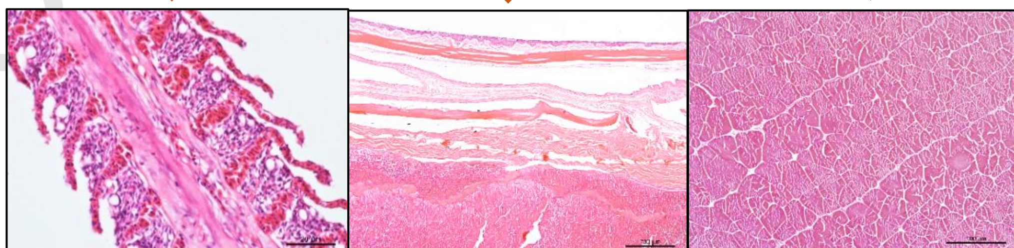

GILL

SKIN

MUSCLE

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Figure 1: A diagrammatic illustration of the steps involved from preparation of fixatives to preparing histological slides until the slides are ready to be viewed. (1a) Adult fish that was ready to be dissected. (1b) Samples that being immersed in 10% Formalin solution, 20% Formalin solution, Bouins's solution and Davidson's solution. (1c) Samples that being transfer from 10% and 20% Formalin solution into decalcification solution a (keep for 6 hours) and decalcification b (keep for 10 hours). (1d) Samples that have been stored in 70% alcohol and ready to be sent into machine processor. (1e) Gills sample that have been embedded in paraffin wax. (1f) Skin and muscle tissue sample that have been embedded in paraffin wax. (1g) Gill, skin and muscle tissue that has been sectioned, stained and mounted. The histological slide is ready to be viewed. (1i) Histology of gill, muscle and skin observed under microscope.

3.6 Statistical Analysis

Statistical analysis between groups was done by using SPSS (version 25) with Kruskal-Wallis test.

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4.0 RESULTS

4.1 Histology of gills

A gill filaments consist of two rows of secondary lamellae (SL) that are aligned along both sides of the primary gill lamellae (PL). The primary gill lamellae covered by epidermal tissue (stratified epithelium) containing mucous cell (MC), chloride cell (CC) and pavement cells and got central venous sinus (VS). The secondary lamellae are lined by squamous epithelium and many capillaries split by pillar cells (PC) that run parallel along the surface.

Each gill of each fish was divided into 6 groups which are group A (10% Formalin solution with Decalcification treatment a), group B (10% Formalin solution with Decalcification treatment b), group C (20% Formalin solution with Decalcification treatment a), group D (20% Formalin solution with Decalcification treatment b) group E (Bouin's Solution) and group F (Davidson's Solution).

From the histopathological findings observed in the gill, lifting of the cartilage was observed in fish fixed with 10% Formalin solution and also Davidson solution. Almost well-preserved gills filament was observed in Bouin's solution and also 20% Formalin solution. On the other hand, the secondary lamellae disorganization can be observed in fish fixed with 20% Formalin solution. Meanwhile, the intensity of H&E stain was observed the best in 20% Formalin compared to another group. For lifting of respiratory epithelium parameter, this parameter was observed in the sample of fish fixed with Davidson solution. Lastly, few ruptures of lamellae epithelium were observed occur in the fish fixed with 20% Formalin solution.

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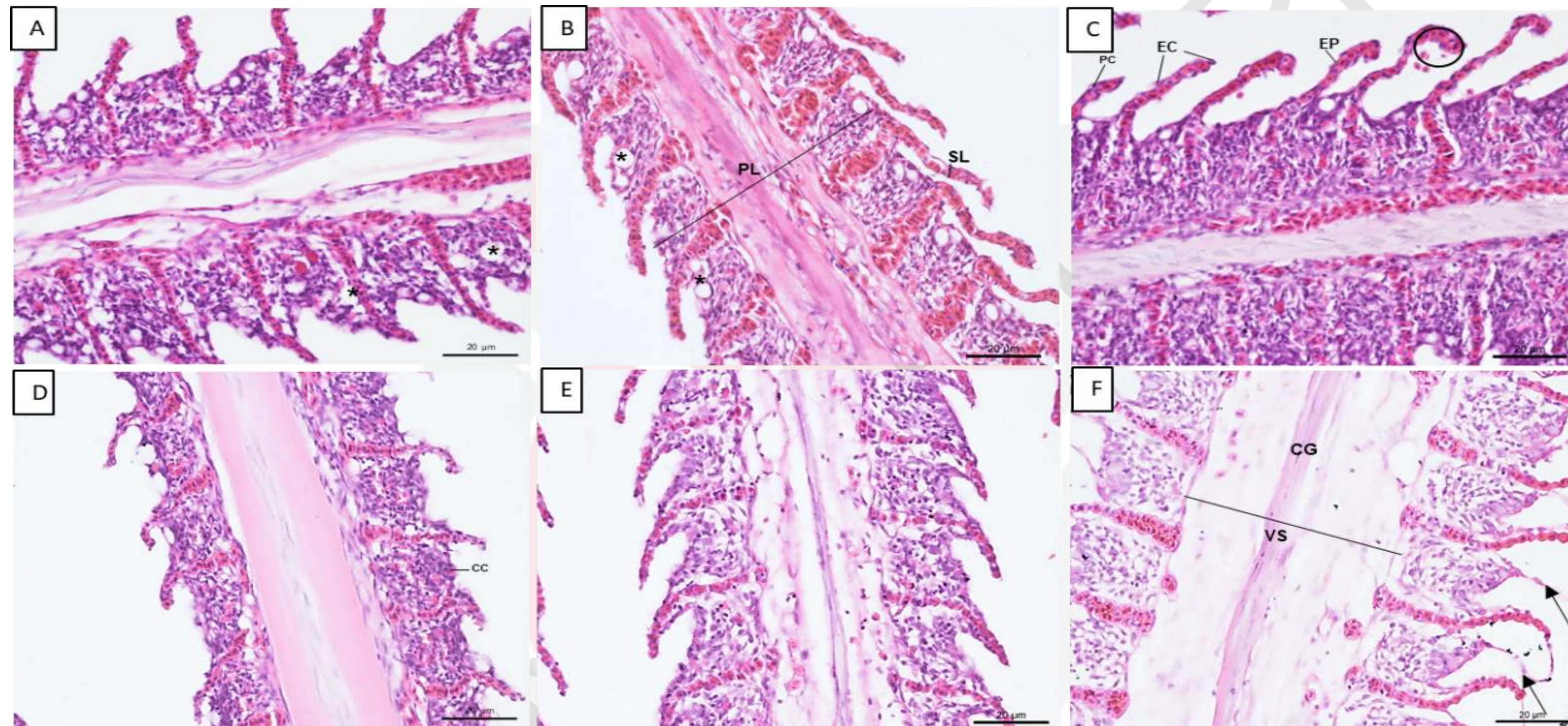
A = Fixed in 10% formalin solution + 6 hours' decalcification treatment, B = Fixed in 10% formalin solution + 10 hours' decalcification treatment, C = Fixed in 20% formalin solution + 6 hours' decalcification treatment, D =Fixed in 20% formalin solution + 10 hours' decalcification treatment, E = Fixed in Bouin's solution, F = Fixed in Davidson's solution, PL= Primary lamellae, SL= Secondary lamellae, CG= Cartilage tissue

Figure 2: Photomicrographs of gills of fish fixed with six group of fixatives. (1A) Lifting of the cartilage was observed shown by the arrow heads. (1B) Almost well-preserved gills filament was observed. (1C) The secondary lamellae are disorganized. (1D) The intensity of H&E stain was quite dark but almost well preserved of secondary gills filament was observed. (1E) Well preserved gills filament was observed. (1F) The epithelium detached artefactually from the capillary. Marked hyperplasia was observed. (H&E Stain; 100x Magnification).



A = Fixed in 10% formalin solution + 6 hours' acid treatment, B = Fixed in 10% formalin solution + 10 hours' acid treatment, C = Fixed in 20% formalin solution + 6 hours' acid treatment, D =Fixed in 20% formalin solution + 10 hours' acid treatment, E = Fixed in Bouin's solution, F = Fixed in Davidson's solution, PL= Primary, PC= Pillar cell, EC= Epithelial cell, EP= Erythrocyte, CC=Chloride cell, CG= Cartilage tissue, VS= Central venous sinus

Figure 3: Photomicrographs of gills of fish fixed with six group of fixatives. (1A). Space formation between the epithelial cell are shown by the asterisk (*). (1B) Marked space formation between the epithelial cell was observed. (1C) The black circle shows rupture of the epithelial cell of the secondary lamellae. (1D) The secondary lamellae were quite short and fragile (1E) Well preserved gills filament was observed with slight lifting of cartilage tissue was observed (1F) The epithelium detached artifactually from the capillary and the intensity of RBC cytoplasm was quite pale in colour. (H&E Stain; 400× Magnification).

4.2 Histology of skin

The outermost layer of skin is known as the epidermis (E), which are lined by stratified squamous epithelium. A high number of goblet cells (GB) are well distributed among the epithelium. The dermis (D) is located in between the epidermis and the underlying body musculature (M). The superficial layer of the dermis is covered by scales which are arranged in scale pockets made up of loose connecting tissues. The loose connective tissue is also known as stratum spongiosum while the dense connective tissue is known as stratum compactum.

Each skin of each fish was divided into 6 groups which are group A (10% Formalin solution with Decalcification treatment a), group B (10% Formalin solution with Decalcification treatment b), group C (20% Formalin solution with Decalcification treatment a), group D (20% Formalin solution with Decalcification treatment b) group E (Bouin's Solution) and group F (Davidson's Solution).

From the histopathological findings observed in the skin, a very intact and perfect layered of skin was observed in the fish fixed with 20% Formalin with acid treatment b. Other than that, space formation between the stratum spongiosum and the scale was observed in the fish fixed with 10% Formalin solution with acid treatment a and b, 20% Formalin solution with acid treatment a and Davidson's solution. Slight lifting of the epithelial cell of epidermis was observed in the fish fixed with 10% Formalin solution with acid treatment b, 20% Formalin solution with acid treatment a and also in Davidson's solution. Lastly, for parameter space formation between dermis can be observed in the fish fixed with 10% Formalin with acid treatment a and also in Bouin's solution.

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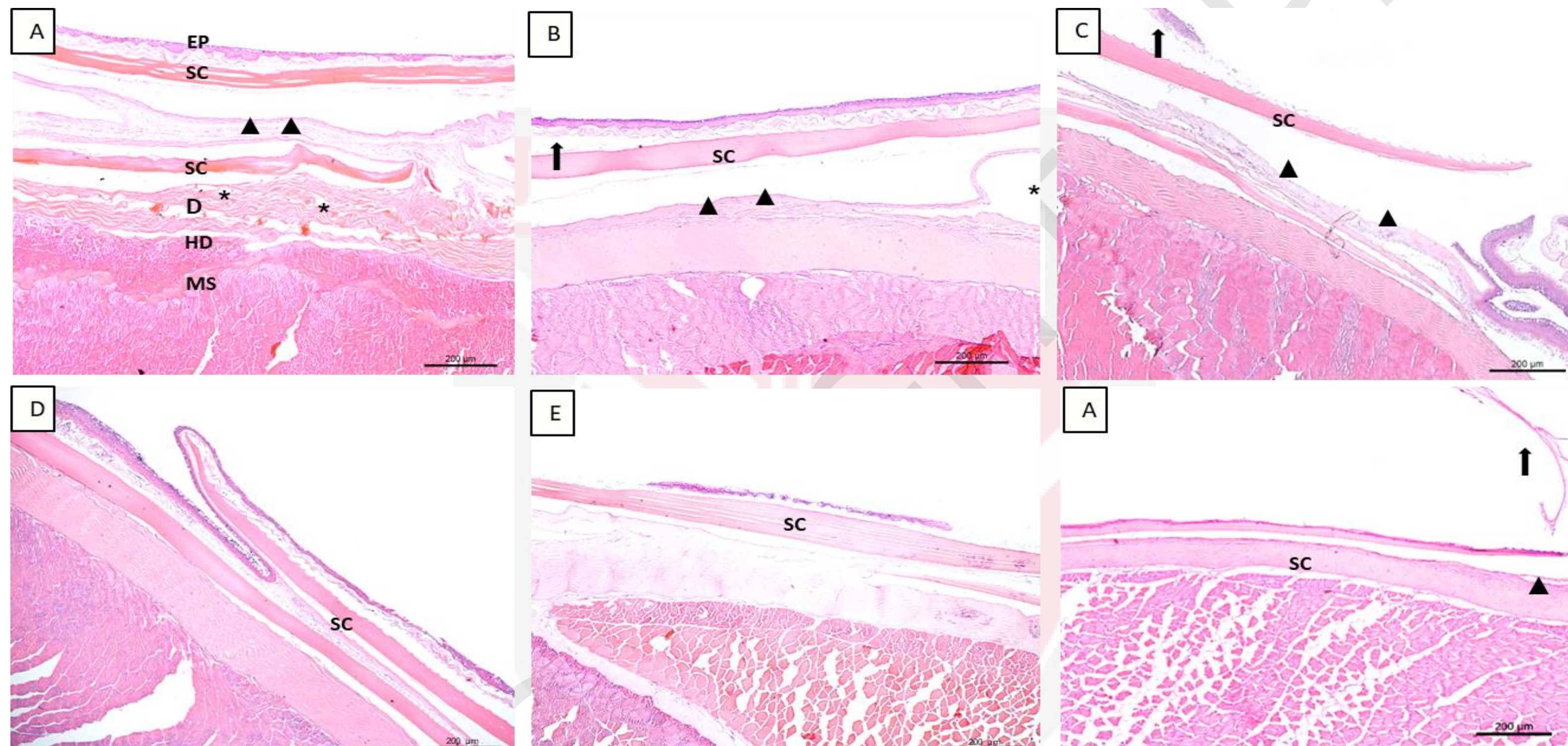

A = Fixed in 10% formalin solution + 6 hours' acid treatment, B = Fixed in 10% formalin solution + 10 hours' acid treatment, C = Fixed in 20% formalin solution + 6 hours' acid treatment, D =Fixed in 20% formalin solution + 10 hours' acid treatment, E = Fixed in Bouin's solution, F = Fixed in Davidson's solution, EP= Epidermis, DR= Dermis, SC= Scales, HD= Hypodermis, MS= Muscle.

Figure 4: Photomicrographs of the skin tissue of fish fixed with six group of fixatives. (1A). Space formation between the stratum spongiosum and the scale are shown by the arrow head. (1B) Space formation between dermis and slight lifting of the epithelial cell of epidermis was observed shown by arrow. (1C) Lifting of the epithelial cell of the epidermis was observed with marked space formation between dermis. (1D), (1E) Well intact skin layer was observed. (1F) Marked lifting of the epithelium of the epidermis was observed. (H&E Stain; 40× Magnification).

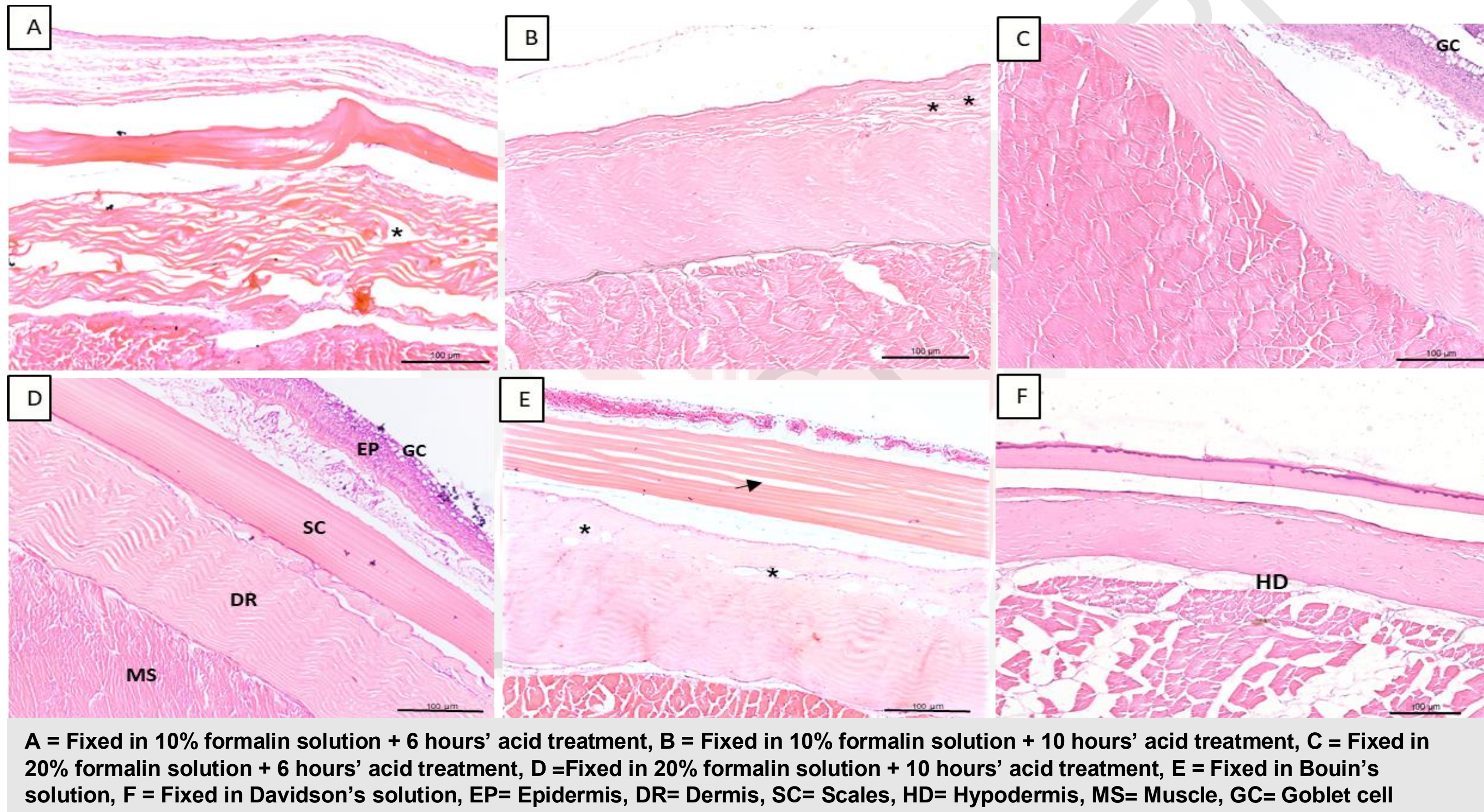

Figure 5: Photomicrographs of the skin tissue of fish fixed with six group of fixatives. (1A). Marked space formation between the stratum compactum was observed shown by asterisk (*). This picture also represents low quality of intensity of H&E stain. (1B), (1C) Presence of slight space formation between the stratum compactum was observed. (1D) Well intact skin layer was observed (1E) There are space formation observed in between the scale, shown by arrow. (1F) Marked lifting of the epithelium of the epidermis was observed. (H&E Stain; 100× Magnification).

4.3 Histology of muscle

Muscles consist of elongated muscle fibres, held together by connective tissues. Many individual muscles show an arrangement of fibres into bundles separated from each other by connective tissue. Each myofibril is composed of two types of short myofilaments.

Each muscle of each fish was divided into 4 groups which are group A (10% Formalin solution), group B (20% Formalin solution), group C (Bouin's solution) and group D (Davidson's solution).

From the histopathological findings observed in the muscle, the space formation between muscle fibre obviously occurred in 10% Formalin solution and slightly occurred in Bouin's solution and also Davidson's solution. Meanwhile for parameter gap formation between myofibrils, it was observed in fish fixed with Bouin's solution and also Davidson solution. Lastly for parameter intensity of H&E stain, the quality is lowest in the fish fixed with 20% Formalin solution.

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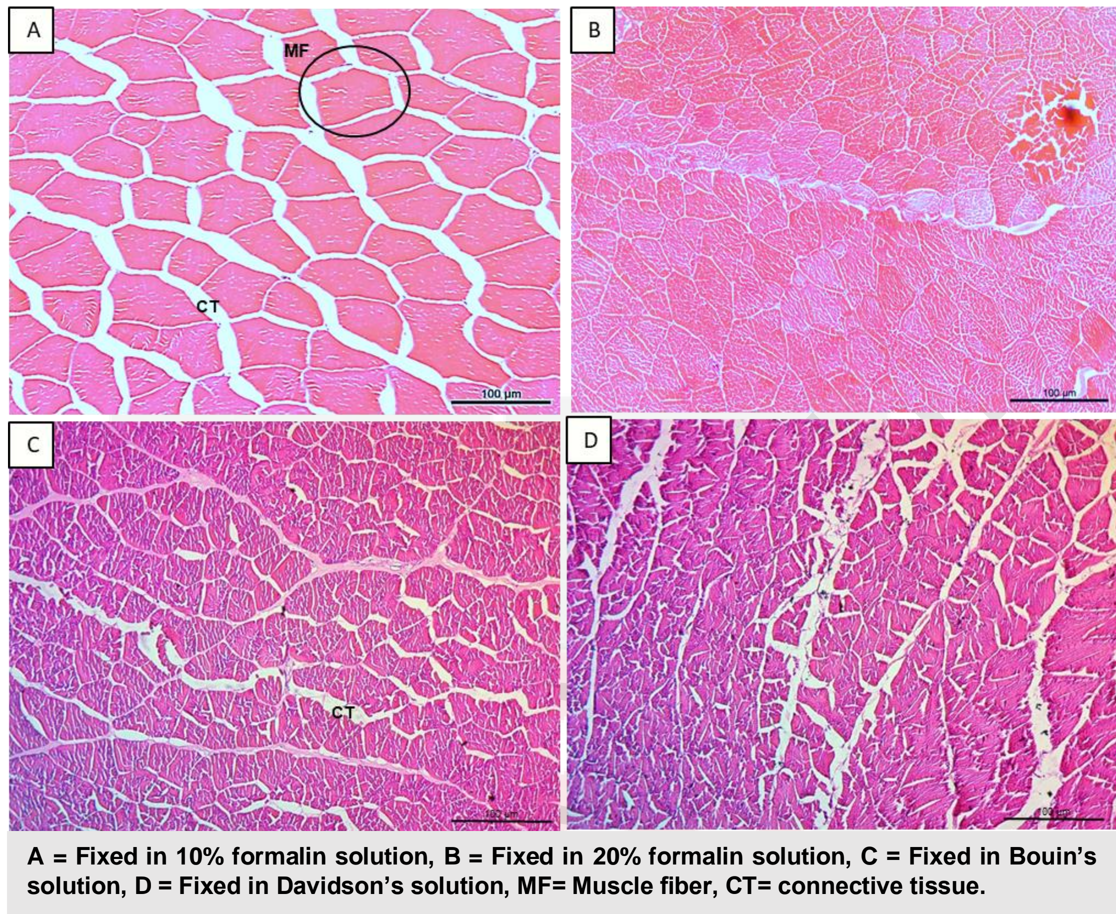

A = Fixed in 10% formalin solution, B = Fixed in 20% formalin solution, C = Fixed in Bouin's solution, D = Fixed in Davidson's solution, MF= Muscle fiber, CT= connective tissue.

Figure 6: Photomicrographs of the muscle tissue of fish fixed with six group of fixatives. (1A). (1B). (1C) Presence of slight space formation between the muscle fibre was observed shown by arrow head. (1D) Presence of moderate space formation between the muscle fibre was observed. (H&E Stain; 100× Magnification).

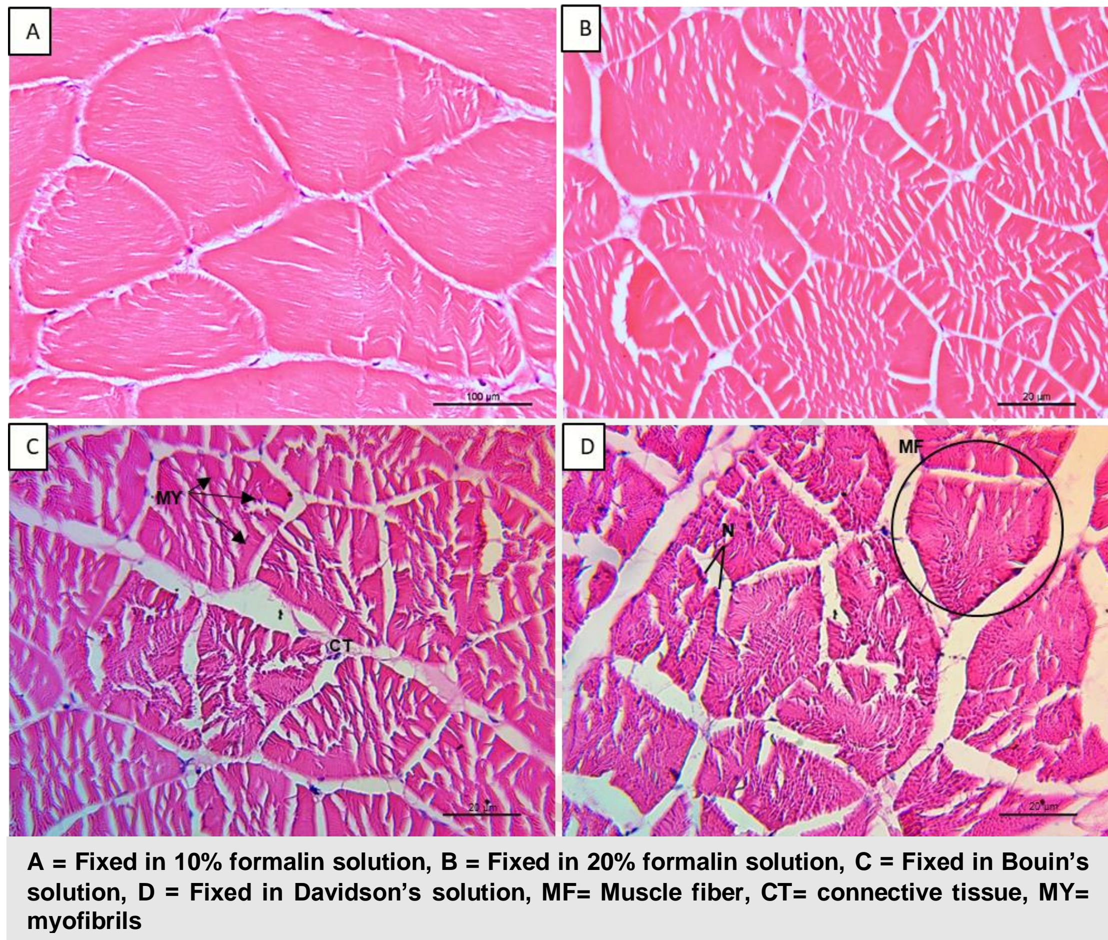

Figure 7: The histological findings observed in the muscle tissue of fish fixed with six group of fixatives. (1A). (1B). (1C), (D) Moderate gap formation between single myofibrils was observed. (H&E Stain; 400× Magnification).

4.4 Decalcification treatment

There are two type of decalcifiers, acid decalcifiers and also chelating agents. Acid decalcifiers are divided into two which are strong (inorganic) acids and weak (organic) acids. Weak acids examples are aqueous formic acid, formic acid with formalin and also buffered formic acid. In this reasearch, we were using formic acid formalin and also buffered formic acid.

4.5 Statistical analysis and graphs of gill

From the statistical analysis, it is revealed that among 4 groups, out of 8 parameters of gills which are the intensity of H&E stain, lifting of the cartilage tissue, disorganisation of secondary lamellae, space formation, lifting of respiratory epithelium, rupture of lamellae epithelium, nuclear morphological clarity, ease to section a specimen, all parameters except disorganization of secondary lamellae and rupture of epithelium were significantly different ($p<0.05$) (See A1 in the appendix A).

There were several histological fixatives alteration that can be observed in all groups of fixatives. Scoring was done for each parameter and the mean score from the statistical data was taken as shown in Figure 8 – Figure 12.

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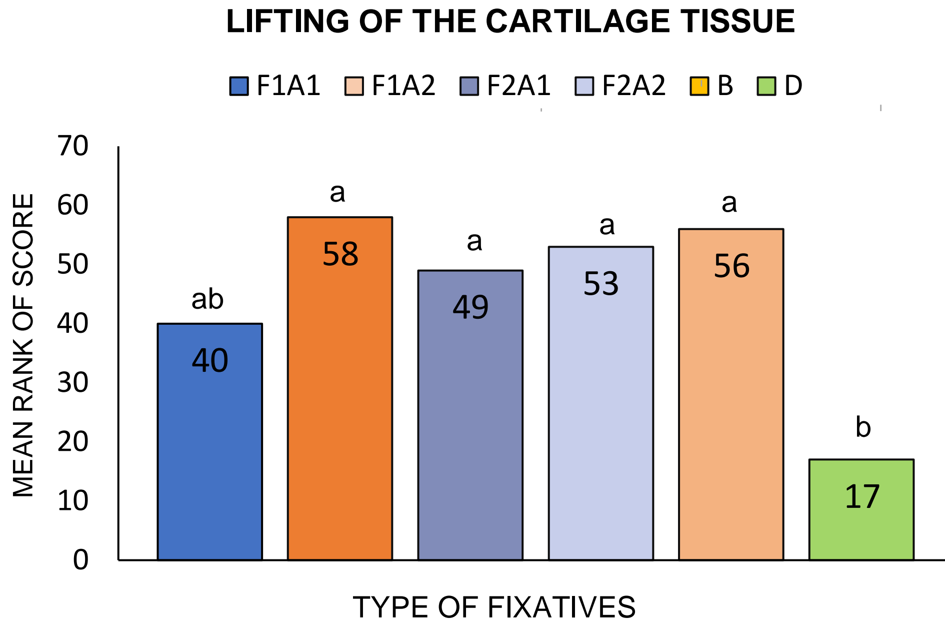

Figure 8: The mean rank of score for parameter lifting of the cartilage tissue.

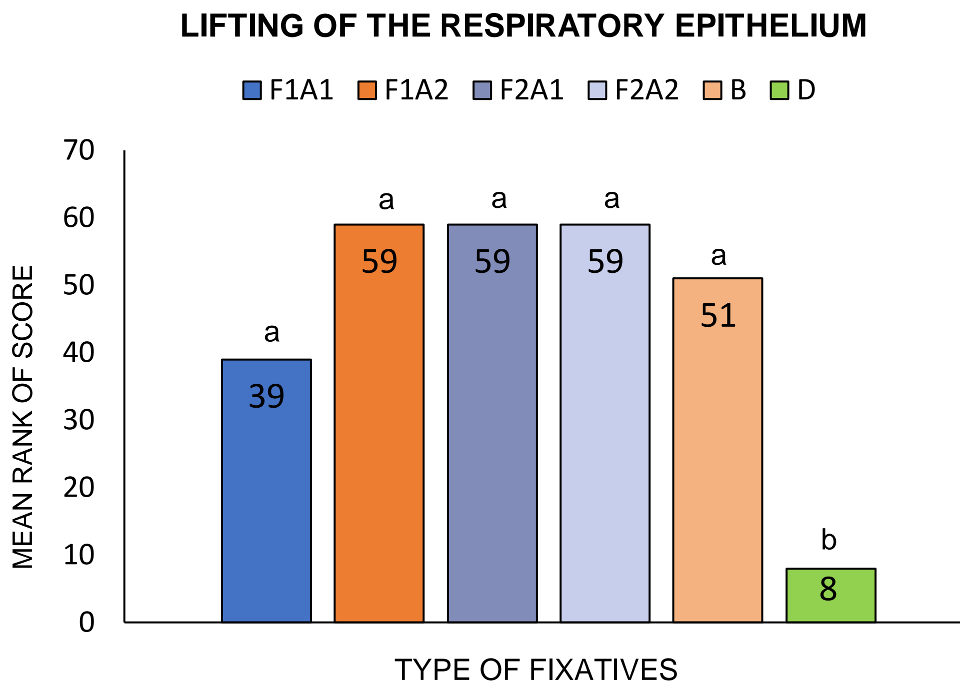

Figure 9: The mean rank of score for parameter lifting of the respiratory epithelium.

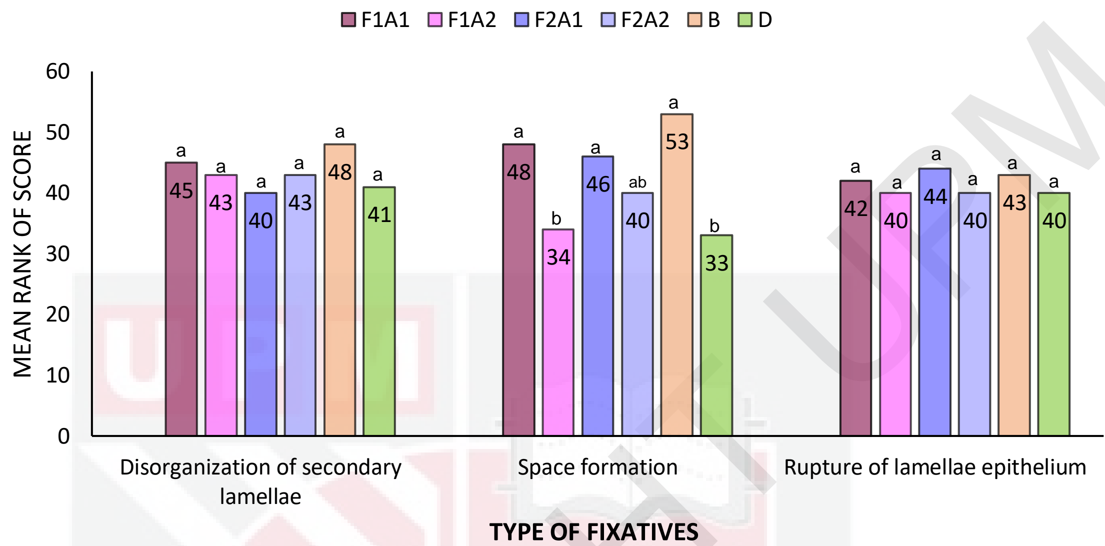

Figure 10: The mean rank of score for parameter disorganization of secondary lamellae, space formation and rupture of lamellae epithelium.

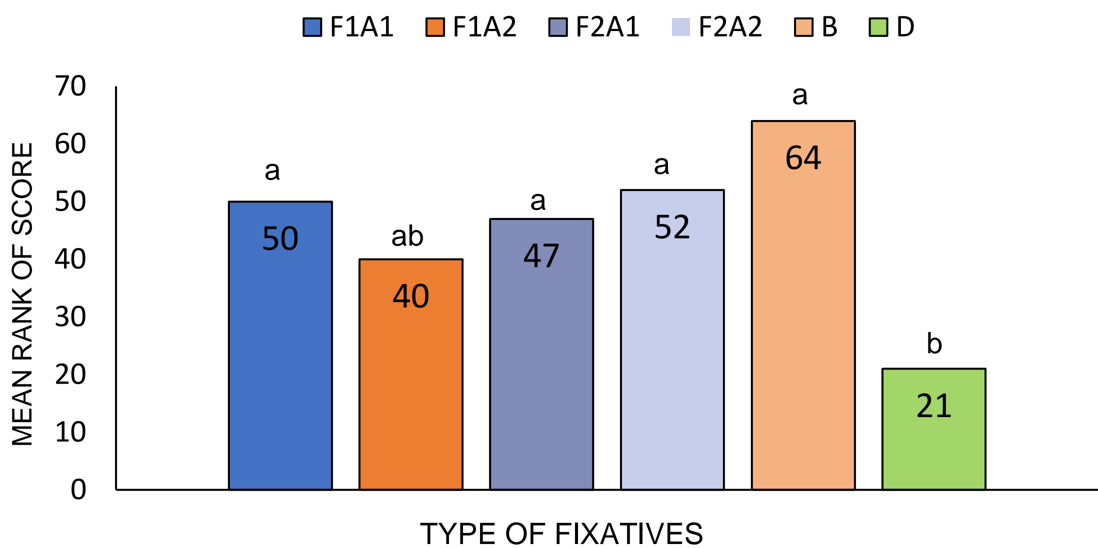

Figure 11: The mean rank of score for parameter nuclear morphological clarity.

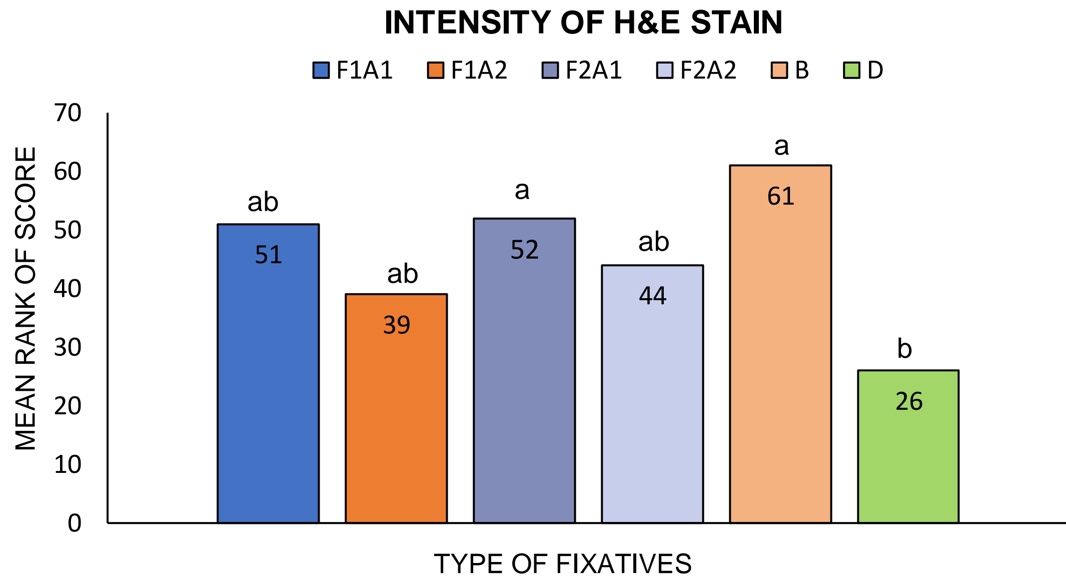

Figure 12: The mean rank of score of intensity of H&E stain.

4.6 Statistical analysis and graphs of skin

For skin tissue, out of 4 parameters analysed which are the intensity of H&E stain, lifting of the epithelium, space formation between dermis and ease to section a specimen, all parameters were statistically significant ($p<0.05$) (See A2 in Appendix A).

Several histological fixatives alteration was observed in almost all groups of fixatives. Scoring was done for each parameter and the mean score from the statistical data was taken as shown in Figure 13 and 14.

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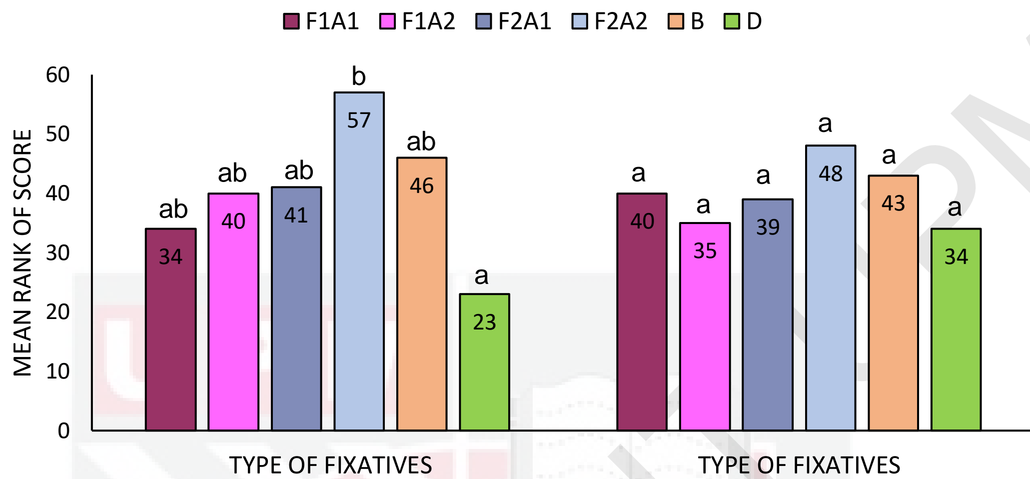

Figure 13: The mean rank of score of lifting of the epithelium and space formation between dermis.

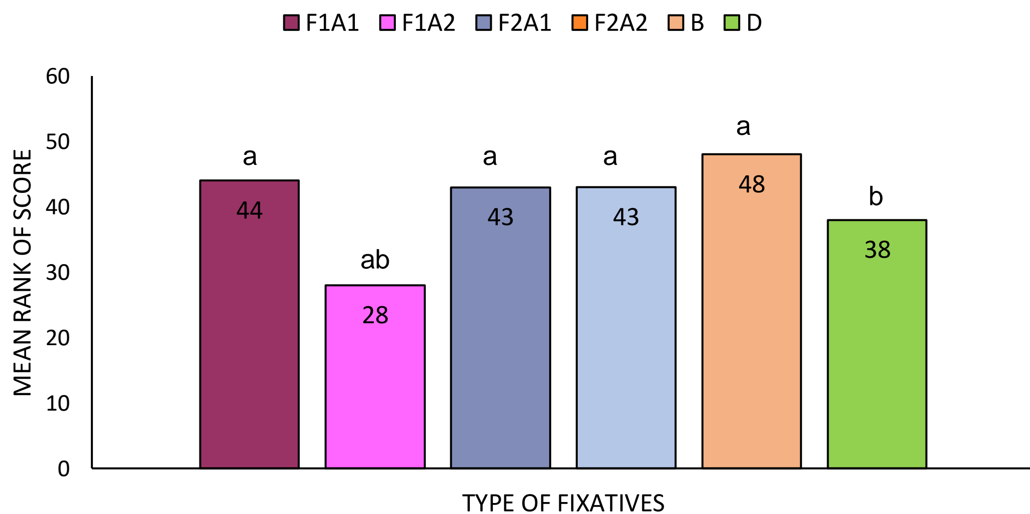

Figure 14: The mean rank of score of intensity of H&E stain.

4.7 Statistical analysis and graphs of muscle

For muscle tissue, out of 4 parameters of muscle which are the intensity of H&E stain, space formation between muscle fibres, gap formation between myofibrils and ease to section a specimen, two of the parameters which are the intensity of H&E stain and space formation between muscle fibres were significantly different ($p<0.05$) (See A3 in Appendix A).

Several histological fixatives alteration was observed in almost all groups of fixatives. Scoring was done for each parameter and the mean score was taken as shown in Figure 15 and 16.

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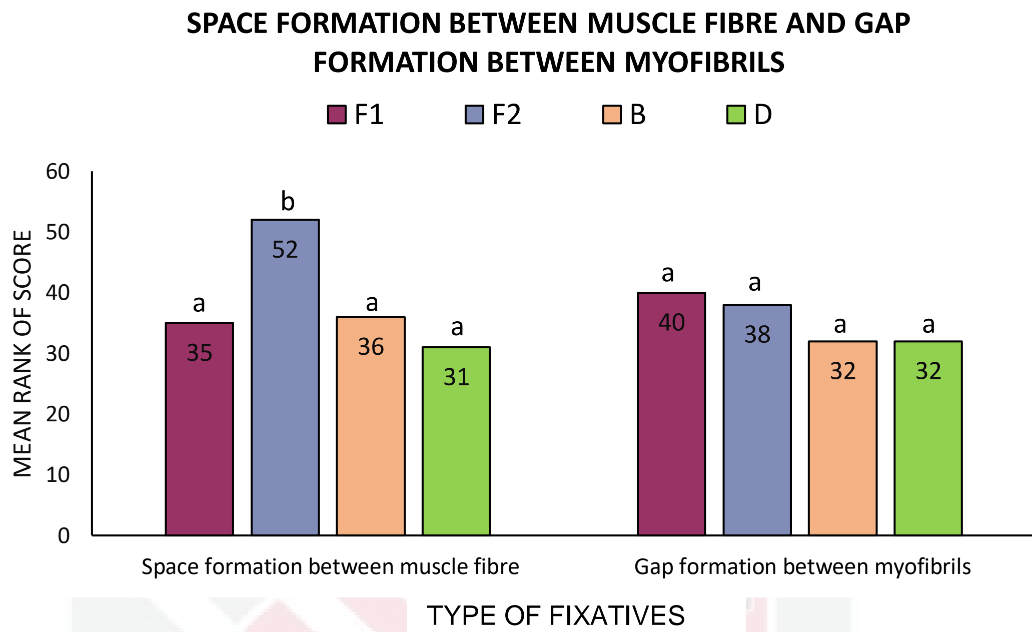

Figure 15: The mean rank of score of space formation between muscle fibre and gap formation between myofibrils.

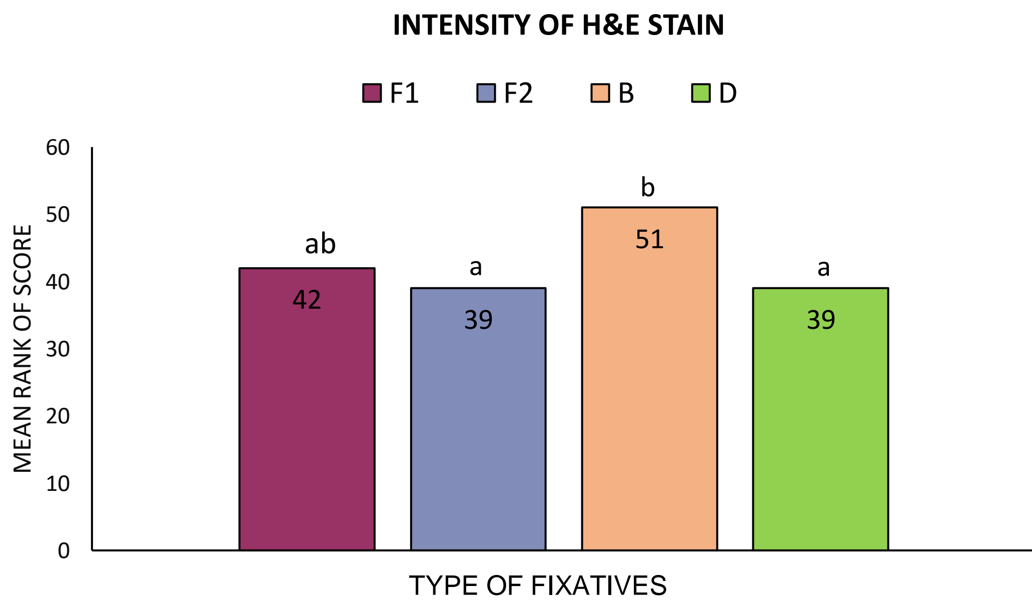

Figure 16: The mean score of intensity of H&E stain.

4.8 Statistical analysis and graphs of decalcification treatment

For the decalcification treatment parameter, there are significantly different ($p<0.05$) between acid treatment 2 and 1 used to decalcify the gills organ, based on the ease to section a specimen parameter evaluated.

Scoring was done for each parameter and the mean score was taken as shown in Figure 17.

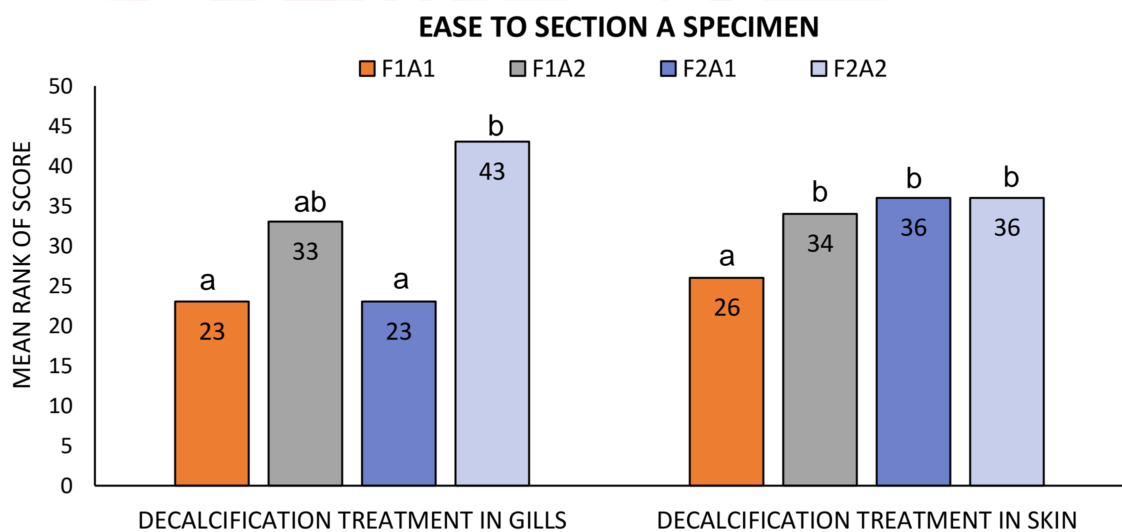

Figure 17: The mean rank of score of parameter ease to section a specimen.

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5.0 DISCUSSION

The goal of fixation is to preserve cells and extracellular materials to resemble *in vivo* tissue architecture as closely as possible (Bancroft and Gamble, 2008). Fixation halts the degradation of tissue by preventing autolysis and by killing microorganisms; while stabilizing the macromolecular structure of the tissue by crosslinking, to allow for downstream tissue processing steps (Bancroft and Gamble, 2008). Additionally, fixation and fixative choice can have a significant impact on subsequent tissue processing steps, particularly sectioning and staining (Bancroft and Gamble, 2008). With all these factors in mind, we set out to determine the most suitable fixative to preserve gill, skin and muscle tissues of Red Tilapia (*Oreochromis spp*.).

In this research, alteration of tissues of the gill that were statistically significant (p<0.0.5) among four types of fixatives are lifting of cartilage tissues, lifting of respiratory epithelium, space formation between epithelium, nuclear morphological clarity and intensity of H&E stain. Lifting of the cartilage tissue of the gill and lifting of the respiratory epithelium may be due to swelling effect of acetic acid and shrinking effect of picric acid that causes the cartilage to be separated from surrounding epithelium (Pallav et al., 2016). Bouin's solution consisting of picric acid, acetic acid and formaldehyde, it has both a coagulative as well as the cross-linking effect on proteins. In particular, the penetration of picric acid into the tissue is slow and it coagulates proteins but has no known chemical interaction with them. The acetic acid that presents in Bouin's solution penetrates relatively quickly and opposes the tissue shrinkage caused by the picric acid that also present in it (Howat et al., 2014). This explained why lifting of the respiratory epithelium only obviously occur in Davidson's

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solution and not in Bouin’s solution. This is supported by Miki et al, 2018 which states that gill filaments and lamellae that fixed with Davidson’s solution were poorly separated, and the gill structure was not so clear.

For nuclear morphological clarity parameter, there are significant differences between Davidson’s solution with other fixatives used which due to the lowest scored obtained by the fixative (Davidson’s solution). The same finding was observed by Peterson et al., (2013) and Miki et al., (2018) which states that fixatives that containing acetic acids such as Davidson’s have been reported to cause erythrocyte lysis but Natural Buffered Formalin inhibits the lysis of RBC. Ulucan et al., (2019) also states that in H&E staining, Carnoy's solution, that contains chloroform, ethanol and glacial acetic acid (which almost same as Davidson’s solution) has been found to provide a more severe nuclear staining, which is thought to be due to the agglutination of nucleic acids. In addition, it was found that the most ideal H&E staining was with Natural Buffered Formalin and the other fixatives produced different intensities. Bouin’s and Davidson’s solutions also reported showing strong cytoplasmic staining with eosin. (Ulucan et al., 2019)

Meanwhile, alteration of tissues of the skin that gives significance differences ($p<0.0.5$) among four types of fixatives are lifting of the epithelium of the epidermis, space formation between dermis and intensity of H&E stain. The lifting of the skin epithelium in Bouin’s or Davidson’s solution may due to the shrinking effect of picric acid and ethanol that can cause artifactual space formation in between the epidermis (Miki et al., 2018). For lifting of the skin epithelium in tissue that fixed in Natural buffered formalin, it may also be due to steps in tissue processing. This is supported by Taqi et al., 2017 which states that prolonged floating of sections on the water bath may

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cause tissue to expand, become distorted and give epithelium an acantholytic appearance mimicking edema.

For the histological alteration of tissues of muscle, parameter space formation between muscle fiber and the intensity of the H&E stain gives significance differences ($p<0.05$) among those four types of fixatives. As we know skeletal muscle contain muscle glycogen. There are a large number of fixative recipes proposed for glycogen preservation and most investigators prefer fixatives containing alcohol or picric acid, e.g. Rossman's and Bouin's solutions (Manns, 1958). Meanwhile, fish pathologists have favoured Gendre's and Davidson's fluids (Hinton et al. 1984, Hampton et al. 1985 and Bullock 1989). Our findings are therefore in contrast, as Bouin's and Davidson's fluid being the least effective. Attempts to explain this apparent discordance are again dubious, but fixatives with precipitating properties may be inferior to pure cross-binding ones with regard to loss of cytoplasmic contents when the glycogen load is considerable, as was the case in this investigation.

Next, for the parameter intensity of H&E stain, a significance differences result was obtained for organ gills, skin and muscle among four types of fixatives used. Our finding was supported by Bird et al., (2012) which states that in a histological examination, different fixation applications can result in different staining results. In determining H&E staining quality we evaluated for colour and tissue clarity. The nuclear clarity and staining were good in tissues that were adequately fixed with Natural Buffered Formalin at 4 °C (Copper et al., 2018).

Lastly, for the objective to determine the best decalcification treatment used to decalcify gill and skin tissues of the tilapia fish is solely depends on the criteria of ease to section a specimen. In this research, there are differences in the score of ease to

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section a specimen between acid treatment 1 and 2 (in 10% Buffered Formalin) and acid treatment 1 and 2 (in 20% Formalin) in gills and skin, there are significance results showed between decalcification treatment 1 and 2 in both fixatives (10% Formalin and 20% Formalin) in gills. This finding was supported by Bancroft (2018), which states that tissues that decalcify with prolonged decalcifying solution give better result during trimming and sectioning.

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CONCLUSION

From the results, we reject the null hypothesis, where, there were significant differences among four types of different fixatives and the decalcification treatment used on the histological structure of Red Tilapia (*Oreochromis spp*). Based on the results, Bouin's solution has the highest total mean score of all parameters for gills sample, but the 10% Formalin and 20% Formalin solution are also the suitable fixatives that used for gill. Meanwhile, for skin and muscle tissues, 20% Formalin recorded the highest total mean score, but the 10% Formalin and Bouin's solution also can be used for skin, while for muscle, Bouin's solution also suitable to use as fixatives. For the best decalcification treatment, the most suitable decalcifiers used is buffered formic acid for 10 hours based on the ease to section a specimen parameter evaluated. In conclusion, different fixatives will yield different results in different type of tissues.

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RECOMMENDATIONS

For further studies, we highly recommended to include other factors affecting the quality of fixation such as the duration to immerse the organs into the fixatives, the temperature of the fixatives and also its pH. Another recommendation for the study is also to study the interaction between tissue processor technique that give effect on the quality of the histological slide, such as the process of embedding, slicing and also staining.

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APPENDIX A

Test Statistics[a,b]								
	Intensity of H&E stain	Lifting of the cartilage tissue	Disorganisation of secondary lamellae	Space Formation	Lifting of respiratory epithelium	Rupture of lamellae epithelium	Nuclear morphological clarity	Ease to section a specimen
Kruskal-Wallis H	19.054	29.396	6.306	29.399	68.827	5.712	26.928	49.815
df	5	5	5	5	5	5	5	5
Asymp. Sig.	0.002	0.000	0.278	0.000	0.000	0.335	0.000	0.000

a. Kruskal Wallis Test

b. Grouping Variable: Fixatives Protocol

*significant level at p<0.05 (Kruskal-Wallis Test)

A1: Result of Kruskal-Wallis Test of gill

Test Statistics[a,b]				
	Intensity of H&E stain	Space formation between dermis	Lifting of the epithelium	Ease to section a specimen
Kruskal-Wallis H	23.944	55.282	16.302	37.584
df	5	5	5	5
Asymp. Sig.	0.000	0.000	0.006	0.000

a. Kruskal Wallis Test

b. Grouping Variable: Fixatives protocol

*significant level at p<0.05 (Kruskal-Wallis Test)

A2: Result of Kruskal-Wallis Test of skin

Test Statistics[a,b]				
	Intesity of H&E stain	Space formation between muscle fiber	Gap formation betweeen myofibrils	Ease to section a specimen
Kruskal-Wallis H	14.863	20.919	5.480	2.105
df	3	3	3	3
Asymp. Sig.	0.002	0.000	0.140	0.551

a. Kruskal Wallis Test

b. Grouping Variable: Fixatives protocol

*significant level at p<0.05 (Kruskal-Wallis Test)

A3: Result of Kruskal-Wallis Test of muscle

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