ADDIS ABABA UNIVERSITY
SCHOOL OF MEDICINE
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Evaluating the Efficacy of Turmeric Solution as Cytoplasmic Stain in Routine Staining Procedure:

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This is to certify that the thesis prepared by Belaynesh Zewdu, entitled: ***Evaluating the efficacy of turmeric solution as cytoplasmic stain in routine staining procedure, comparative study*** and submitted in fulfillment of the requirements for the Degree in Master of Science (Histotechnology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

AAU = Addis Ababa University

C.longa = curcuma longa

Eosin –B = eosin bluish

Eosin-Y = eosin yellowish

EPA = Environmental Protection Agency

FMoH = Federal Ministry of Health

FFPE= Formalin Fixed Paraffin Embedded

GIT = Gastrointestinal tract

H&T= Hematoxylin & Turmeric

H&TM= Hematoxylin & Turmeric with mordant

H and E = Hematoxylin and eosin

MSDS = Material Safety Data Sheet

OSHA = Occupational Safety and Health Authority

P^H = Power of Hydrogen "or" Potential of Hydrogen."

PI = Principal Investigator

RBC = Red Blood Cell

SoM = School of Medicine

SOP = Standard Operating Procedure

SPSS = Statistical Package for Social Science

TASH = Tikur Anbesa specialized hospital

Operational Terminologies

Blocks: cassettes filled with paraffin wax holding infiltrated and embedded tissues in side.

Histopathology: a branch of pathology concerned with the tissue changes characteristic of disease.

Sectioning (Microtomy):-preparation of thin sections from fixed and embedded tissue blocks.

Staining:-artificial coloration of a substance to facilitate examination of tissues, microorganisms or other cells under the microscope.

Mordant: Substance that causes certain staining reactions to take place by forming a link between the tissue and the stain.

Nuclear staining: the intensity of basophilia in a cell.

Cytoplasmic staining: the intensity of eosinophilia in a cell.

Uniformity of staining: devoid of patchy staining and out-of-focus areas throughout the section.

Clarity of staining: devoid of cloudiness throughout the section.

Crispiness of staining: being able to see well delineated nuclear membranes and sharply stained condensed chromatin against an unstained nucleolus.

Background: the obscuring of any tissue part by stain other than the tissue nature

Diagnosable section: is a section that has been reported as adequate for three or more of the above parameters by the pathologists.

Abstract

Background: *histopathology is a gold standard method in pathology where tissue samples are grossed, processed, cut, stained, mounted and microscopically evaluated. Samples of tissue are taken from patients and then prepared using appropriate staining protocols to detect and diagnose the disease. Hematoxylin and eosin is the most pronounced technique. Eosin is a synthetic dye derived from fluorescein. Eosin is hazardous to humans and animals. With the increasing awareness of green earth it is advisable to use more of eco-friendly and biodegradable material which can be effectively achieved by the use of natural dye from plants and other natural sources. Turmeric available as curcuma longa (domestic) is believed to have staining property.*

Aim: *This study aimed to evaluate the efficacy of turmeric solution as cytoplasmic stain in hematoxylin and eosin staining procedure for substitution of hazardous eosin.*

Method: *prospective cross-sectional method and convenience sampling technique was used at Tikur Anbesa Specialized hospital pathology department in August 2020. Forty tissue blocks were used. From each block three sections were taken at 4µm thickness, a total of one hundred twenty sections grouped into three and stained turmeric with mordant (H&TM), turmeric without mordant (H&T) and conventional eosin (H&E) staining methods. Staining was evaluated by three histopathologists independently, using standard checklist. Slides were scored using six parameters: Nuclear, cytoplasmic, uniformity, clarity, crispness and background of staining. Slides that satisfied at least four of the aforesaid parameters were considered adequate for diagnosis. Z- test and chi-square test were used to compare the difference among the three methods, p-value < 0.05 considered significant.*

Result: *TM stained slides were adequate for diagnosis was comparable with that of the conventional eosin method. There was no statistical significant difference among pathologist as well as the methods p-value was >0.05(0.123). On average from a total of 120 stained slides, 112(93.3%) stained slides were adequate for diagnosis and 8 (6.7%) inadequate. but adequacy and substituting capability of HT with that of eosin showed high discrepancy in adequacy. There was statistical significant difference among methods p- value was<0.05(0.00) .On average from a total of 120 stained slides 69(57.7%) were adequate for diagnosis and 51(42.5%) inadequate.*

Keywords: health hazardous, staining, substitute, turmeric, mordant.

1. Introduction

1.1 Background

Histopathology has been the gold standard for tissue evaluation and assessment.In histopathology samples of tissue were taken from patients and then prepared using different chemicals of staining protocols to detect and diagnose the disease with by aid of an instrument microscope [1]

Stains are substances used to give color to tissue or cells. Staining is a technique, following sectioning and commonly used in the medical diagnosis of tumors in which a dye color is applied on the anterior and posterior border of the sample tissues to locate the tumorous or diseased cells or other pathological cells. [2, 3]

The presence of charges, H+ in dyes determines attractive and repulsive properties. Dyes can be acidic or basic depending on PH concentration. Acidic dyes have ph. <7 while basic dyes have ph. >7. [4]

Paraffin sections which are unstained cannot be evaluated, that is why necessary to apply coloring reagents (mostly chemicals) to stain tissue structures. Hematoxylin and Eosin (H&E) is the routine staining used to study histopathology changes in tissues and organs from animals in toxicity studies. [5]

Hematoxylin and eosin (H&E) technique was introduced in 1875.It is the most widely and commonly used stain. More than a century to identify tissue characteristics and structures morphologies those are needed for tumor diagnosis. Hematoxylin dye that stains the nuclei giving it a bluish color while eosin stains the cell's cytoplasm giving it a pinkish stain. [6, 7, 8]

In histopathology, stained cytoplasm of a cell provides an over-view of shape, size and changes within the cells giving distinct indications for diagnosis. [9]

Dye is natural or synthetic substance used to impart colour to tissue in the diagnostic field of histopathology and histochemistry. The availability of biomedical dyes in the market is smaller and with lower than expected quality. Unsatisfactory results emerge due to quality and

availability of Hematoxylin and eosin solutions as we use what we get, although it activates to find solution for draw backs. [10]

Dyes produced from animals or vegetables in the absence of chemical processing are called natural dyes which are mostly derived from insects, minerals and plants, like curcumin from turmeric plant. Natural dyes are Subtle, plentiful, safe, ecological friendly, unsophisticated, harmonized with nature, simple to dispose, range from soft to brightest color, and leave reaction during preparation. These along with social, economic, and environmental benefits accelerated the demand for natural dyes. Natural dyes may or may not request mordant to form bond with tisue. [11, 12]

Interest of using synthetic dyes depreciated due to their toxicity and hazardous nature to humans and environment. Greatest option for this is to use natural dyes which are safe to environment and harmless. Toxic and allergic reactions along with the formulations of environmental standards grow the interest to use natural dyes instead of synthetic ones which is like 150 yrs. back.[13]makeshift people's awareness to ecological and environmental initiatives to focus on natural dyes in spite of synthetics.[11,14,15]

Eosin is a synthetic dye derived from fluorescein and it is a member of xanthene family of dyes which are eosin-Y, eosin -B, phloxin-B and fluorescein. It is yellowish –red crystalline powder, can be soluble in water or alcohol. Eosin has a molecular mass -691.85 g/m and molecular formula -$C_{20}H_6Br_4Na_2O_5$. Eosin dyes are bromine derivative of fluorescein which has two very closely related dyes commonly known as Eosin yellowish (Eosin Y) and Eosin bluish (Eosin B). [16]

Eosin Y is a tetrabromo derivative chemically known as disodium 2-(2, 4, 5, 7-tetrabromo-6-oxido-3-oxo3H-xanthen-9-yl) benzoate. Fluorescein in the eosin Y molecule called fluorochrome exists in two forms; one is the more stable quinoid form which is colored and fluorescent while the other one is lactone form which is colorless and non-fluorescent. [16]

Potential Health Effects of Eosin are nausea, vomiting, eye irritation, dermatitis, pneumoconiosis Bromides induce sedation, irritability, agitation, delirium, memory loss, confusion, disorientation, forgetfulness (aphasias), dysarthria, weakness, fatigue, vertigo, stupor, coma,

decreased appetite, diarrhoea, hallucinations, bronchoderma, coryza, Ataxia and gehyperreflexia have also been observed. [17, 18]

Various studies have demonstrated the use of natural dyes for use in plant as well as animal histological studies and have found satisfactory results in comparison to the synthetic stains used. [19]

The worldwide shortage of eosin and its hazardous effects of chemicals and synthetic dyes led to the development of alternative organic and eco-friendly dyes from these natural sources. The use of these non-allergic, non-toxic, and biodegradable stains has become the need of the hour due to the increased environmental awareness. Limited studies have been performed to determine the efficacy of turmeric as a stain for formalin fixed paraffin embedded sections that are routinely stained with Hematoxylin and Eosin. [20]

Turmeric which has a botanical name Curcuma longa and Curcuma aromatica is herbaceous plant, member of the Zingiberaceae (ginger) family and native of South Asia. It is grown in tropical countries Ethiopia, India, Pakistan, Myanmar, Chile, Peru, etc., have also synonyms diferulomethane, Indian saffron, curcuma & haldi. Turmeric (Curcuma longa.) is One of exported spice in Ethiopia, Southwest Ethiopia produce this spice as a cash crop and many lively hood had been depend on it for a living. Ethiopia is the biggest producer and exporter of turmeric in Africa. Its Average price of per kg is USD 1.10. [21]

The plant Turmeric available as curcuma longa (domestic) has long been use in subcontinent as spice and favoring agent in most food preparation. Turmeric (Ird in Amharic) is one of the most favored spices of Ethiopian housewives and is the main ingredient of the local sauce *alicha-wot.* [22]

There are approximately 110accepted Curcuma species; however the exact number of species is still debated. Only about 20 species have been studied phytochemically and Curcuma longa is the most chemically investigated species.The genus is best known for being an essential source of coloring and flavoring agents in the Asian cuisines, traditional medicines, spices, dyes, perfumes, cosmetics, and ornamental plants. [23, 24]

9-Turmeric (Curcuma longa) have a chemical formula Curcumin [1, 7-bis (4-hydroxy-3 methoxyphenyl)-1, 6 heptadiene-3, 5-dione] is an orange-yellow component of turmeric (Curcuma longa), a spice often found in curry powder which have amolecular formula $C_{21}H_{20}O_6$. [24]

The Physical Description is: solid Orange-yellow crystalline powder, with color form Orange-yellow, crystal powder; gives brownish-red color with alkali solutions; light-yellow color with acids solutions. Solubility: Slightly soluble (hot water), Insoluble in cold water. Very soluble in ethanol, acetic acid. [27]

Chemically, curcumin is a diarylheptanoid, belonging to the group of curcuminoids, (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) which are natural phenols responsible for turmeric's yellow colour. It contains flavonoids, which are typically polyphenolic compounds. Phenols are acidic, due to their ability to release the hydrogen from their hydroxyl group, hence the ability of C. longa to stain the basic parts of the cell. Attracted uses as cytoplasmic stain in this study. In addition to the rhizome's richness in curcuminoid pigments (6%) and essential oils (5%), it also contains 69.43% carbohydrate, 6.30% protein, 3.50% mineral and other important nutrients on dry weight basis. [21, 26, 27]

Curcuma longa L. is a popular natural drug, traditionally used for the treatment of a wide range of diseases.Turmeric possesses several biological activities including anti-inflammatory,antioxidant,anticancer,antimutagenic,antimicrobial,anti-obesity,hypolipidemic,cardioprotective, and neuroprotective effects.[28.29]

The ethanolic extract of Curcuma longa is relatively harmless by acute and Sub-chronic oral administration. Therefore, this extract does not present any risk of toxicity for consumers at all doses used. [29]

1.2 Statement of the Problem

Histopathology diagnosis requests production of well stained slides which has the capacity to depict cellular details. The synthetic dye, Eosin, is a cytoplasmic stain used in routine staining of H&E method. Substitution of such dyes with natural ones is the major issue in hazard reduction. For these, many trials have been tried; however still eosin is in use. [13, 30]

The Cost of dyes like eosin used in diagnostic staining incurs service cost inflation. Moreover, as many developing countries can no longer afford the ever‑increasing cost of synthetic dyes ground, for instance, the cost of eosin is seven fold to that of turmeric. [31, 32]

Employees' safety is an important issue in histopathology laboratories. However routinely used eosin has hazardous effect due to its bromine and fluorescein content .It causes inflammation to skin, irritate eyes, throat, inhalation, allergic reaction if inhaled, ingested or in contact with skin. [5, 33, 34]

Most of the histopathology laboratory in developing countries has depended on expensive imported reagents for use in their laboratories. From these staining chemicals the synthetic dye eosin was the most imported item which many pathology centers come up in shortage.

There is also an empirical gap in many studies done on substitution of eosin. Majority of researchers used very small type and number of tissues with discrepancy in tissue variety. On top of that, there are also drawbacks in their method of analysis. Majority of studies checked only the staining ability instead of substitution capacity

Studies done on natural dyes using human tissues samples were not able to fully substitute eosin. Hence staining is still dependent on the presence of eosin. Hence, there is gap in terms of assessing natural dyes to substitute with eosin with natural extracts of plants that are both eco-friendly and cost-effective has gained importance. [19]

Therefore, there is no better way than replacing the expensive, hazardous and toxic eosin with Safer, cheaper and eco-friendly substitute. This research aims to assess if turmeric c.longa is a good substitute for eosin in routine staining procedure to make histopathology laboratory a safer place to work and create less polluted environment in general

1.3 Significance of the Study

The finding of this study will help laboratory professionals to work in hazard minimized areas.

With the increasing awareness of a green earth, finding a safe the synthetic eosin substitute is an ideal option and advisable to use non-allergic, non-toxic, more of environment‑friendly and biodegradable natural dye.

Natural dyes confirmation for substitution of imported synthetic dyes will help minimize the cost of service production and shout down times with in sighting the new area of market for producers of the plant.

This finding will also fill some empirical gaps which were investigated during revising literatures like sample size determination, types of tissues to be included and the criteria for analyzing substitution capacity of chemicals.

Finding substitution for eosin will help the academicians, researchers and students as an input for further research may be as a counter stain in other special stains.

2. Literature Review

The study was done by Archana Sudhakaran. et al: in Department of Oral Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Dharwad, Karnataka, India in 2018 Natural stains Zingiber officinale Roscoe (ginger) and Curcuma longa L. (turmeric) – A substitute to eosin Extracts of fresh rhizomes of Zingiber officinale Roscoe and Curcuma longa L. were obtained by dissolving them in alcohol, which was used to stain sections of 25 cases. Observer 1 compared the staining intensity, while observers 2 and 3 assessed preset parameters at regular intervals and subjected to Kappa statistics and Mann–Whitney U- test. The results Z. officinale showed better staining intensity and specificity to the cytoplasm and basic components of connective tissue as compared to C. longa. Crispness (P = 0.01) and background staining (P = 0.05) showed a significant difference. Shelf life of Z. officinale was better than C. longa. [19]

The study was done by Department of Oral Pathology, MS Ramaiah University of Applied Sciences, Bengaluru, Karnataka in India in 2017 researchers Marin Abraham et al., Histologic Comparison between Curcumin Stain and Synthetic Eosin. This study was used the two extraction methods, Curcuma longa was dried, powdered and extracted by maceration and soxhlet techniques. A total study samples 40 tissue sample 20 normal tissues and 20 pathological Tissues (OSCC-Oral Squamous Cell Carcinoma) sections were stained turmeric with and without the mordant. Stained slides were evaluated and compared by three pathologists (observers).The staining intensity, efficacy of prepared turmeric stain with that of eosin were compared by appreciating the respective tissue structures stained by both the components. The statistical analysis was carried out by Chi-square test. The result was reported as statistically significant results were observed in sections stained by maceration technique than that of the soxhlet. Sections stained with prepared Turmeric extract with mordant stain fetched comparatively better result (2%, p=0.6530) when compared to turmeric stain, The Hematoxylin and Turmeric (H&T) sections in which Mordant was incorporated with Turmeric fetched better results. Comparable results were obtained in normal and pathologic tissue sections stained with H&T (TEM) with conventionally employed H&E stain. Conclusion: Turmeric extract can be used as an adjunct stain to eosin, a synthetic stain. [20]

Another study was conducted by Rubina MP.et al: in the Histopathology lab of a tertiary health care center in South India in 2020, Assessment of Staining Quality of Curcumin as a Substitute

for Eosin in Hematoxylin and Eosin Staining in Histopathology. This study was prepared the turmeric powder and used maceration technique. During the study period from five tissue types 100 samples used For each staining method 100 sections were prepared from 20 collagen tissues, 20 epithelial tissues, 20 smooth muscle tissues, 20 bony tissues and 20 adipose tissues. The staining qualities were assessed by one experienced pathologist and the results were scored as excellent, good and poor. After entering the scores in Microsoft excel, the results were statistically analyzed. The result was reported as 95% of slides stained with H and C staining were good and 100% slides are excellent with H and E staining for collagen ($x2=80 p<0.001$). For epithelium H and E staining, 45% of slides were graded as excellent and 52.5% as good and 2.5% as poor. In epithelium H and C staining 87.5% slides were graded as good and 12.5% as poor ($x2=24.167$ $p<0.001$). For muscle tissue H and E staining gave excellent score in75% of slides, good score in 25% of slides. For H and C staining 72.5% slides were good and remaining were graded poor in muscle tissue ($x2=50.256 p<0.001$). Most of the slides stained with H and E and H and C staining for bone tissue were graded as poor, only 20% slides with H and E staining showed good quality($x2=10.141 p=0.001$).In adipose tissue100% slides with H and E staining showed excellent staining, only 30% slides in H and C showed good score($x2=43.077 p<0.001$). they concluded that H and C staining gave comparable results with H and E staining in all the five types of tissue sections studied (p value<0.05) with intense affinity on collagen and muscle fibres, Curcumin is a safer and cheaper alternative to Eosin stain in histology and histopathology sections. [26]

Another study was done in Department of Oral Pathology and Microbiology, Chhattisgarh Dental College and Research Institute, Rajnandgaon, Chhattisgarh, India in 20017 by researcher Suryawanshi, et al : Curcuma longa extract – Haldi: A safe, eco- friendly natural cytoplasmic stain. The result was staining ability of epithelium, keratin, muscles, adipocytes, blood vessels and RBCs with curcumin was almost as good as eosin. Whereas for collagen fibers, cartilage and bone staining ability of curcumin was not as good as eosin with $P < 0.05$, which suggests that curcumin stains these structures in a different manner imparting a yellowish hue to them. The P value obtained was statistically insignificant ($P > 0.05$). The study was concluded that eosin is the most efficient counterstain for hematoxylin, turmeric can also be used as an alternative for eosin. [32]

Similar study was conducted by Department of Oral Pathology and Microbiology, Kothiwal Dental College and Research Centre, Moradabad 244 001, Uttar Pradesh, India, by researcher Sachin Kumar, et al.: Use of Curcuma longa extract to stain various tissue samples for histological studies. The study was retrospective a One year old wax blocks of human oral tissues obtained from both healthy and pathological individuals were procured from archives of Department of Oral Pathology and Microbiology. Statistical analysis revealed that, overall staining ability of eosin is better than turmeric. However, interestingly collagen, muscle fibers shows $P < 0.05$, which suggest that turmeric dye stain these structures in a remarkably different and distinct pattern giving deep yellowish orange color when compared to other tissues as suggested by their P values, which were 0.143 and 0.280 respectively. They conclude that turmeric can be used as a counterstain after hematoxylin, in place of synthetic toxic eosin dye. [35]

Carlo C Bondoc conduct a research in Philippines in 2018: Curcuma longa Linn rhizome extract as an alternative stain for histological studies. This research claimed that different levels (15, 20, 25g) of turmeric dissolved in 100 ml of 70% alcohol would affect staining plant and animal tissues. Specifically, one way ANOVA was used to assess the significant variations among the different independent groups which are the 15, 20, and 25 grams of turmeric stain and the chemically synthesized stain on various plant and animal tissues. the results of the analysis of variance on the staining capacity of 15, 20, and 25 grams of C. longa in 100mL of 70% alcohol solution. The F-value obtained in the calculation is less than the F-critical value. Therefore, it can be drawn out that the variations in the concentrations of alternative stain do not produce significantly different staining effects on plant and animal tissues. But among the different prepared concentrations, the 15 grams yield more excellent staining result. Therefore, this quantity of turmeric may be set as the standard amount in preparing an alternative stain out of turmeric unless further related studies on the masses of turmeric will be conducted. [37]

The researcher's team Navya N.et al: study conducted at a rural tertiary care referral institute, PES Institute of Medical Sciences and Research, Kuppam, Andhra Pradesh, India in 2019 evaluate the staining characteristics of kumkum solution as a counterstain and to compare the staining characteristics of kumkum solution and turmeric solution in histopathology sections of cervix tissue. The result reported as the overall performance of kumkum was better than Turmeric and was statistically highly significant ($p<0.001$). [38]

A research team O. G. Avwioro et al. Conducted a research in Nigeria in 2007 Curcuma longa extract as a histological dye for collagen fibers and red blood cells. The rhizomes of turmeric were cut into small pieces and dried then milled to a fine powder. Soxhlet method was used for extraction for turmeric; the study sample was taken Human tissue at post-mortem examination, fixed in 10% formol saline for 24 hr. The result reported as a 1% aqueous solution of C. longa stained red blood cells, collagen fibers, muscle fibers and cytoplasm deep yellow within 5 min. The effects of acidity, alkalinity and mordant on the stain C. longa stained tissue sections yellow in aqueous solutions of acetic acid and ammonium hydroxide (acidic and alkaline solutions, respectively). The addition of potassium alum as a mordant did not improve the staining qualities of C. longa. For this reason, the Allepey cultivar of C. longa was tried as a counterstain because of its high pigment content and used as a substitute for eosin in the hematoxylin and eosin technique. [31]

The study was done by Rosemary B Bassey, et al.: in Nigeria in 2012, Curcuma Longa: Staining Effect on Histomorphology of the Testis: they used the extraction technique of soxhlet method, ethanolic extract of Curcuma longa. It was used to stain histological sections of the testes for 15 minutes. Phytochemical constituents were investigated. Their result was the Curcuma longa dye distinctly stained the seminiferous epithelium and interstitium yellow. Curcuma longa provided a good counter stain for Hematoxylin, taking up the acidic staining characteristics with Hematoxylin staining the basic staining characteristics. Phytochemical screening revealed the presence of saponins, alkaloids, tannins and flavonoids. They concluded that Curcuma longa has good potential for use as a counter stain for Hematoxylin in the staining of tissues in lieu of Eosin. [36]

3. Objective

3.1 General Objective

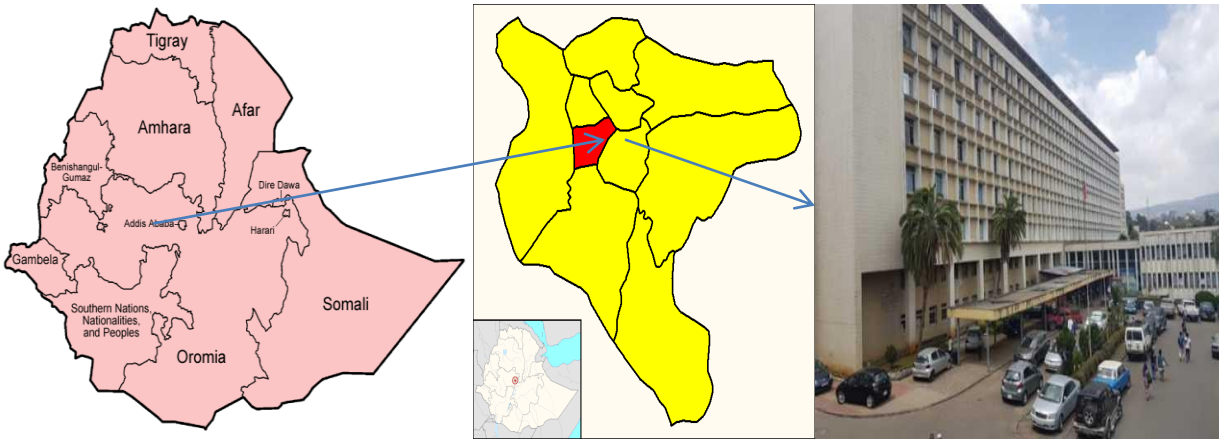
The main objective of this study was evaluating the efficacy of turmeric solution as cytoplasmic stain in routine staining procedure.

3.2 Specific Objective

- To compare the ability of turmeric with that of conventional eosin for ″ nuclear, cytoplasmic and uniformity adequacy.
- To investigate and compare the presence of "clarity, crispness and background of staining of turmeric and eosin
- To determine the substitution capacity of turmeric (with and without mordant) for eosin during staining.

4. Materials and Methods

4.1 Study Area



Ethiopia Adisababa (lideta subcity) Tikur Anbesa hospital

Figure 1**: Addis Ababa university, college of health science, Tikur Anbesa specialized hospital**

The study was conducted at Tikur Anbessa Specialized Hospital (TASH) pathology department which is located at Lideta sub city, Addis Ababa town, capital city of Ethiopia. TASH is one of the tertiary governmental hospitals in the country established in 1965 E.C; it is the largest general public hospital in the country with over 800 beds. Annually, the hospital serves for about 460,000 and 40,000 outpatients and inpatients, respectively (Kefale B. et al., 2018)

In the hospital one of pathology department which serves cytopathology and histopathology services. The patient registration log book of the department shows that in average 170 new biopsy cases per week visit histopathology unit of the department. Per year around 10,000 outpatient cases surgical samples diagnosed in its histopathology unit. All units and departments unit send surgical biopsy samples to the department for diagnosis.

The department also provide postgraduate program in residency and clinical Histotechnology. The department had different types of main histopathological equipment's such as Microtome, Tissue Embedding Console, Cryostat, Tissue Processor, and automated slide stainer, Incubator

and refrigerators. Currently the pathology department had sixteen senior pathologists, one molecular and cancer scientist, sixteen residents, nine senior histotechnologists, one medical laboratory technician three Para technicians and three secretory, two receptionist, two porters and one store kipper.

4.2 Study Design

A comparative cross-sectional study method was conducted to assess the staining and quality of substitute. Checklist was prepared to assess the quality of stained slides and microscopic evaluation by senior pathologists.

4.3 Study Time

The study was conducted from August, 2020 to June, 2021. Total work on sample Preparation, processing, staining, slide distribution and result collection.

4.4 Sample

4.4.1 Source of Sample

The source sample for the study was all biopsies came to TASH received by pathology laboratory, during the study time august 2020

4.4.2 Study sample

The study sample was 40 blocks of tissue samples that had been processed and sent to be embedded, sectioned and stained the following day.

4.5 Sample Size and Sampling Method

According to The national committee for clinical laboratory standard (NCCLS), 2011 guideline recommends a minimum of 40 samples for method comparison, based on this recommendation a total of 40 FFPE tissue blocks were selected by convenient sampling method.

4.6 Eligibility

4.6.1 Inclusion Criteria

All biopsy tissue blocks which were came to TASH, Histopathology laboratory in August 2020 fit for sectioning and whose slides were going to be taken to stain during the study period.

4.6.2. Exclusion Criteria

Biopsy tissue blocks inadequately fixed poorly processed, and tissue/specimen detaches or pulled out and fragmented from the block was excluded.

Also tissue block which were wrongly oriented during embedding and insufficient paraffin wax blocks were excluded.

4.7 Variables

4.7.1 Dependent Variable

- Nuclear staining
- Cytoplasmic staining
- Clarity of staining
- Uniformity of staining
- Crispness of staining
- background

4.7.2 Independent Variable

- Hematoxylin and eosin stains standard.
- Turmeric c. longa with mordant
- Turmeric c. longa without mordant

4.8 Study Procedure and Data Collection

4.8.1 Biopsy Sample Preparation

Different kinds of human tissue samples were used in this study, obtain from lymph node, thyroid tissue, skin, kidney, liver, brain, GIT (gastro intestinal tract),bone tissue, breast, female genital tract, prostate, testicular tissue, gallbladder and pregnant related disorders. The specimens were fixed a fixative reagent 10% formaldehyde.40 tissue were processed routine tissue processing method by "Tissue Tak II"VIP processing machine. Then these samples were embedded in paraffin wax embedding media. Next 40 blocks were sectioned by used a functional semi-automated rotary microtome at 4µm (micrometer). This final step in preparing the samples for staining. Thin sections were placed on to glass slides, dried and stained.

4.8.2 Reagents

Fixative: 10% formaldehyde

Processing reagents: tissue under go to process starting steps from fixation, dehydration, clearing & infiltration pass through reagents respectively formaldehyde, ethanol, xylene& paraffin wax. After complete this process ready to embed and section.

Staining reagents:

H&E stain	H&T stain	H&T+M stain
• Harris hematoxylin • 0.3% eosinY • 1% acid alcohol	• Harris hematoxylin • 15 g.Turmeric.C.longa powder • 70%ethanol • 1%acid alcohol	• Harris hematoxylin • 70%ethanol • 15g.Turmeric C.longa powder • Metallic mordant(potassium aluminum sulfate) • 1% acid alcohol

4.8.3 Preparation of Plant Extract

The rhizomes of turmeric C.longa was purchase from the market of Merkato, cut in to small pieces and dried at 40 C^0 oven for 48 hours. They were milled to form fine powder by using house hold mixer grinder. Then weighted 15 g of powder using an electronic weighing machine.

Solution preparation of turmeric C.longa without mordant

Maceration technique was used, taken 15 g of C.longa powder and dissolved in 100 ml of 70% ethanol (70% ethanol was prepared from absolute ethanol).shake until completely dissolved then left for 48 hours under stable condition. The supernatant solution taken and filtered ready to cytoplasmic stain.

Solution preparation of turmeric C.longa with mordant

Maceration technique was used; taken 15 g of C.longa powder and dissolved in 100ml of 70% ethanol then add potassium aluminum sulfate alum mordant by using the aid of a little heat to dissolve the alum salt. Shake completely to dissolved and left for 48 hours. The supernatant solution was taken and filtered for cytoplasmic stain.

Fig.2 **Preparation of Plant Extract the rhizomes of turmeric**

4.8.4 General Histological Procedure

Table-1Tissue processing schedule

No.	reagents	Schedule time set.	Temperature
1.	4% formalin	1 hour	35
2.	water	5 seconds	35
3.	50% ethanol	1hour	35
4	70% ethanol	1hour	35
5.	80%ethanol	1hour	35
6.	90% ethanol	1hour	35
7.	100% ethanol	1hour	35
8.	100%ethanol	1hour	35
9.	xylene	1hour	35
10.	xylene	1hour	35
11	Paraffin wax	45 minutes	68
12.	Paraffin wax	45minutes	68
13	Paraffin wax	45minutes	68
14.	Paraffin wax	45minutes	68

Staining procedures

From each 40 FFPE tissue blocks three slides were prepared in order to run stain in both three methods, total 120 slides were sectioned by one person with one microtome with the same thickness 4µm.

Then before go to stain the slides were dried in slide warmer settled the temperature at 58-60C^0 above the melting point of paraffin wax.

The slides grouped gassy in to the three staining methods labeled A, B&C next the serial number or code that given by principal investigator (PI). Register the exact label of the slide to a checklist for later cross checking of slide with the staining procedure performed. The three grouped 120 slides were after hematoxylin stain (nuclear stain) stained by conventional cytoplasmic stain Eosin, Turmeric without mordant and Turmeric with mordant (method labeled as 1, 2&3 respectively).

Staining of slides all the three groups until nuclear stain (Harris hematoxylin) used the same procedure,

Deparaffinization ⟶dexylination ⟶ hydration → nuclear stain. For cytoplasm stain conventional eosin (method-1), turmeric without mordant (method-2) and turmeric with mordant (method-3).

For method-1 conventional eosin staining time was given 30-1minute.

For method-2 and method-3 turmeric without mordant and with mordant staining time was given 5-10 minute.

The three methods slides were for each method 40 slides were stained.

For the three methods after cytoplasmic stain washing in tap water ⟶ dehydration⟶ clearing → mounting with DPX same procedure was done.

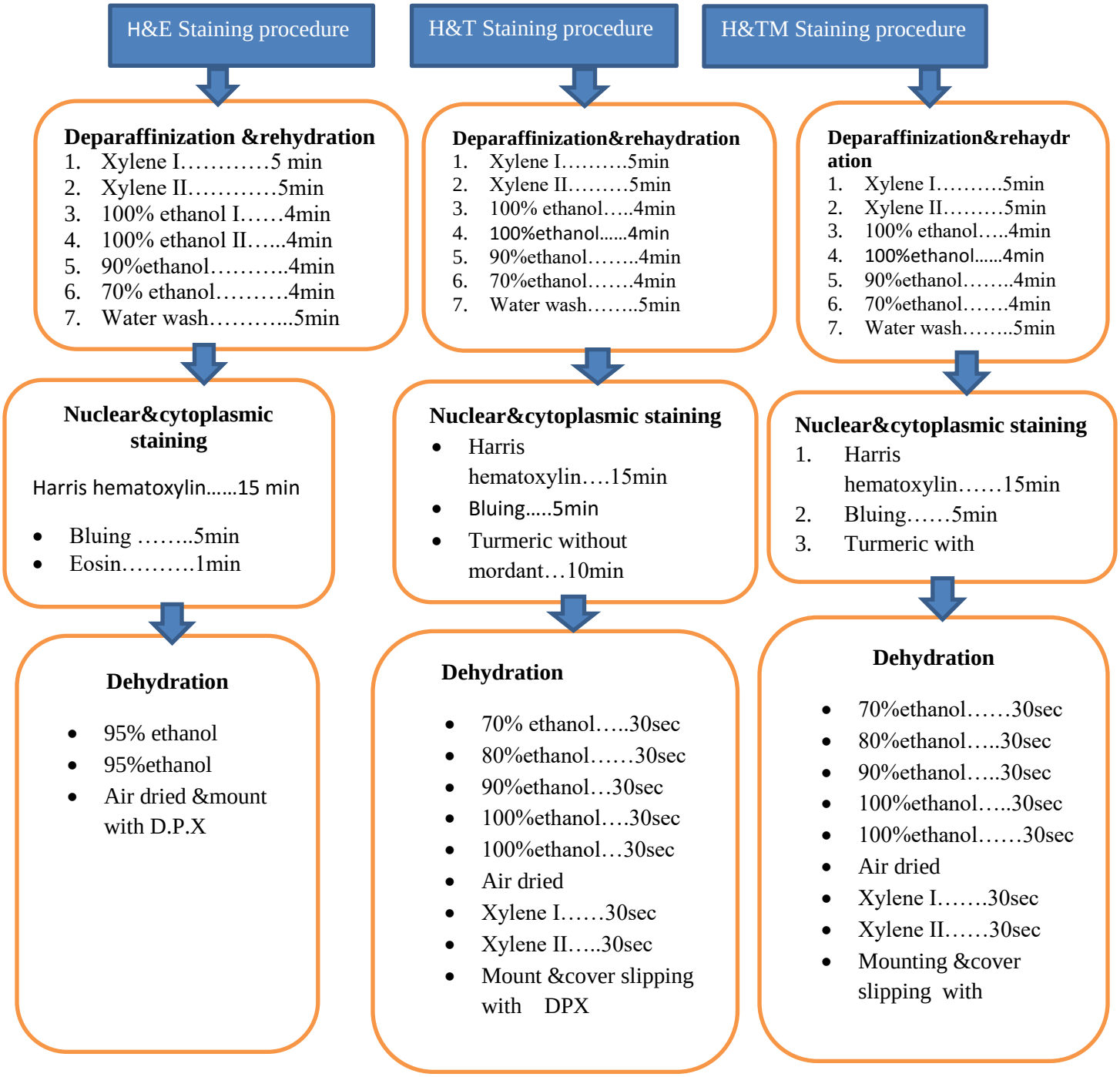


Fig.3 **H&E, H&T and H&TM staining procedure**

4.8.5 Assessment of Stained Sections

All the 120 slides tripled were analyzed by three different senior pathologists referred as pathologist I, II&III. The pathologist used a checklist that contains a scoring system that has been previously similar studies used Marin Abraham et al., Histologic Comparison between Curcumin Stain and Synthetic Eosin .

All the pathologists had no prior knowledge of staining method employed so, the study was blinded to render the observer bias.

The three staining methods conventional H&E, H&T and H&TM were graded based on the following six parameters.

- Nuclear staining, (adequate, score 1,inadequate score 0)
- Cytoplasmic staining, (adequate, score 1, inadequate, score 0)
- Uniformity of the staining (present, score 1, absent, score 0)
- Crispness of the staining (present, score 1, absent, score 0)
- Clarity of the staining (present, score 1,absent, score 0)
- Background of the staining (absent, score 1,present, score 0)

A score of 0& 1 were given to each of these parameters and the slides were graded by adding up the scores. Slides with a score, ≤3 were graded as inadequate for diagnosis, and the slides with score, >4-6 were assigned as adequate for diagnosis. “Z” test and chi-square test were used to compare the three staining methods, $p < 0.05$ considered as significant.

4.8.6 Pilot studies

Before the preparation of the final research slides seven pilot studies were done for the sake of a better/good result. In each pilot study a pair 5(five) slides were taken from different types of tissue section were used. The first and second study was adjusting the concentration of turmeric solution.10g and15g of finely grind powder of turmeric dissolved separately in 100mlof 70%ethanol. The concentration of 10g turmeric powder was weak for staining of cytoplasm and microscopically slides were pale stain.15g turmeric powder was better. Third and fourth study was selection of method(maceration).15g of turmeric powder dissolved in 100ml of 70% ethanol

was centrifuge by centrifuge machine, while the fourth one dissolved and put for 48 hours. Take the supernatant for staining. The supernatant taken from the centrifuge method can stain slides but it takes the time 30 minutes, whereas the maceration method for 48 hours can stain slides within 10 minutes best. Fifth, sixth and seventh study was for arrangement of staining time.by trial and error (5,7&10minuts) were tested. Slides stained within 10 minutes were microscopically best for cytoplasmic stain and 10 minutes was the ideal staining time based on the evaluation of pathologist.

4.9 Data Quality Assurance

4.9.1 Pre analytical

The study tissue blocks taken from 2020 year blocks archive. The tissue blocks were processed by tissue processor "Tissue Tek II" VIP processing machine. All reagents for tissue processing; formaldehyde, alcohol, xylene and paraffin wax were all new (Freshly) prepared.

Expiration date was checked for every powder or solution that has been used in the preparation of reagents. All the staining reagents were prepared by standard protocols (SOP).

Embedding, sectioning, and labeling were done by experienced laboratory technologists, Histotechnologists and the PI. Tissue blocks were cut-sectioned and mounted on slides. For each block, three tissue sections were taken, codes were given for three staining and slides were carefully labeled. During the staining of study samples 120 tissue sections, the PI was able to manage H&E, H&T and H&TM staining using functional timer. Finally, covers slipping of stained slides were properly done. Using a well prepared scoring checklist of having six parameters, each slide was examined under microscope by three pathologists.

4.9.2 Analytical

Temperature set up of the embedding machine, cool plate, dry oven and water bath was checked by Histotechnologists and the PI. Setup of micron meter on the microtome machine was checked by PI for the sake of tissue section thickness, functional timer for staining.

Evaluators/pathologists were blinded for method of staining (experience was 5- 20years)

4.9.3 Post Analytical

Complete report of the data was properly decoded and entered to SPSS version 26 by the PI.

4.10 Statistical Analysis

Data was entered into IBM SPSS version 26 for analysis. Pearson's Chi-square test was performed to show the equivalence/agreement between the matched paired sections (conventional H&E, H&T and H&TM stained sections), $p<0.05$ considered as statistically significant. Most similar researches used "Z" value to look for proportion difference between the three methods stained section. Therefore, in order to compare our research results with those researches, "Z" test was used, $p<0.05$ considered as statistically significant.

4.11Ethical Consideration

Ethical clearance was obtained from departmental research ethics review committee (DRERC) of the department of pathology, collage of health science, Addis Ababa University. The data from Study sample was used for the purpose of this research only. Numerical codes were used all the time. Hence, confidentiality and secrecy were strictly maintained.

5. Result

5.1 Evaluation characterization of H&T, H&TM and H&E stained slides

To compare the staining ability of H&T, H&TM and The conventional method H&E for cytoplasmic stain a total of 40 tissue blocks were included in this study. From each tissue block three sectioned slides were taken.

Over all 40 samples were used for this study. From these 120 stained slides; three for each of them were prepared. All the 120 (40x3) slides were evaluated by three pathologists independently. All pathologists used a checklist that contains six parameters. From the six parameters, those slides that only fulfilled the four or more parameters were considered adequate for diagnosis.

5.2 Overall Frequency of the three stains

Result showed 115(95.8%) H&TM, 61(50.8%)H&T and 119(99.2%)H&E stained sections were adequate while 5H&TM, 59 H&T and 1 H&E stained sections were inadequate for nuclear staining respectively. Cytoplasmic staining was reported adequate for 106 H&TM, 61 H&T and 116H&E and inadequate for 5 H&TM, 59 H&T and 4 H&E stained sections respectively. The pathologists reported 114 H&TM, 97 H&T and 119 H&E stained sections were present and 6 H&TM, 23 H&T and 1 H&E stained sections were absent for uniformity of staining respectively. Clarity of staining was reported the present for 108 H&TM, 85 H&T and 119 H&E and absent for 12 H&TM, 35 H&T and 1 H&E stained sections respectively. Crispness of staining was reported present for 109 H&TM, 43 H&T and 116 H&E and absent for 11 H&TM, 77 H&T and 4 H&E stained sections respectively. Background of staining was reported present for 114 H&TM, 111 H&T and 120 H&E and absent for 6 H&TM, 9 H&T and 0 H&E stained sections respectively.

Table-2 Overall frequency of the three stains

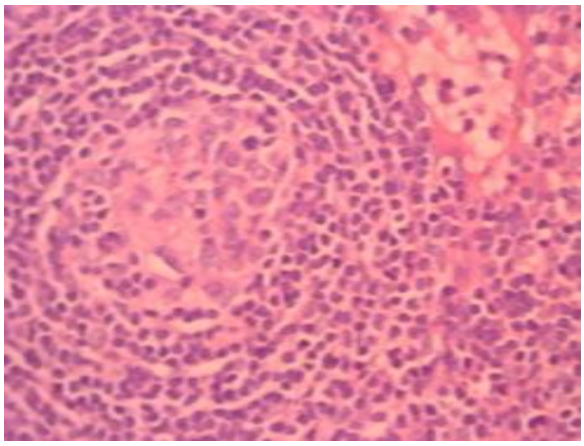
Parameters		H&E	H&TM	H&T
Nuclear staining	Inadequate	1 (0.8%)	5(4.2%)	59(49.2%)
	Adequate	119(99.2%)	115(95.8%)	61(50.8%)
Cytoplasmic staining	Inadequate	4(3.3%)	14(11.7%)	59(49.2%)
	Adequate	116(96.7%)	106(88.3%)	61(50.8%)
Uniformity	Absence	1(0.8%)	6(5.0%)	23(19.2%)
	Presence	119(99.2%)	114(95.0%)	97(80.8%)
Clarity of staining	Absence	1(0.8%)	12(10.0%)	35(29.2%)
	Presence	119(99.2%)	108(90.0%)	85(70.8%)
Crispness of staining	Absence	4(3.3%)	11(9.2%)	77(64.2%)
	Presence	116(96.7%)	109(90.8%)	43(35.8%)
Background staining	Absence	0(0%)	6(5.0%)	9(7.5%)
	Presence	120(100%)	114(95.0%)	111(92.5%)

5.3 Nuclear Staining

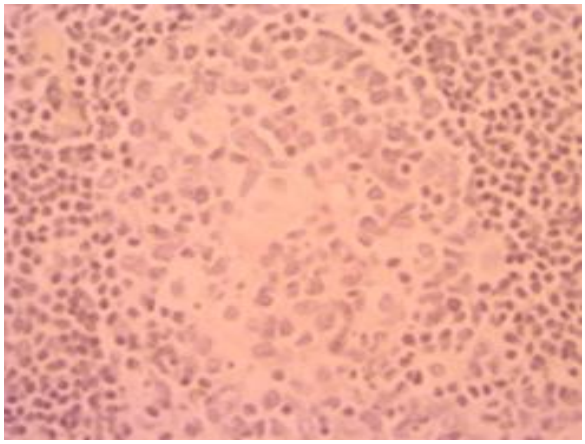
H&TM compared to H&E for nuclear stain and the result showed that from the total 40 paired stained slides 39(97.5%), 40(100%), 40(100%) were adequate by pathologist I, II&III respectively using H&E method. While H&TM method 38(95%), 39(97.5%) and 38(95%) were adequate for nuclear staining by pathologist I, II and III respectively. from 40 stained slides each pathologist evaluated, disagreement reported in 2,1&2 pairs respectively and there was no statistical significant difference among pathologists the chi-square p value >0.05. Within comparison of H&T and H&E pathologist I, II&III reported as 19 (47.5%) 22(55%) and 20(50%) adequate nuclear staining and 21(52.5%) 18(45%) and 20(50%) inadequate nuclear stain respectively. All pathologists favored conventional H&E stained slides over H&T and H&TM stained slides. Both “Z” and chi-square’s indicated no significant difference b/n the conventional & H&TM methods, while showed significant difference with H&T p value <0.05.

Table-3 comparing of nuclear staining of the hematoxylin & eosin with hematoxylin& turmeric with mordant and hematoxylin & turmeric without mordant stained sections.

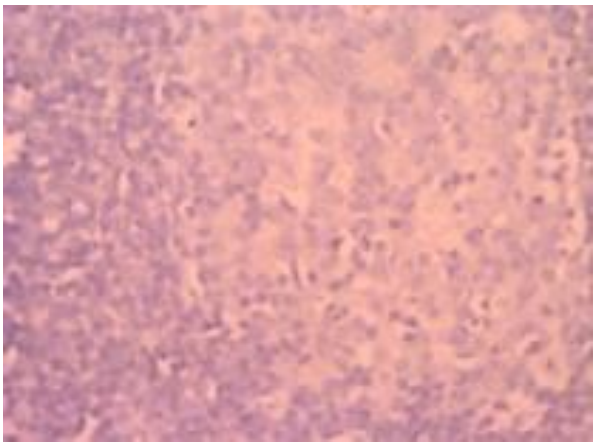
Nuclear staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		inadequate	adequate	total	0.14 (0.556)	inadequate	adequate	total	4 (0.000)
	Inadequate	1	0	1		1	0	1	
	Adequate	1	38	39		20	19	39	
	total	2	38	40		21	19	40	
Pathologist II	Inadequate	0	0	0	0.5 (0.314)	0	0	0	4 (0.000)
	Adequate	1	39	40		18	22	40	
	total	1	39	40		18	22	40	
Pathologist III	Inadequate	0	0	0	1 (0.152)	0	0	0	4.6 (0.000)
	adequate	2	38	40		20	20	40	
	total	2	38	40		20	20	40	



A.H&E(4X)



B.H&TM(4X)



C.H&T(4X)

Fig.4 **Lymph node biopsy photomicrograph shows adequate nuclear staining within the three methods.**

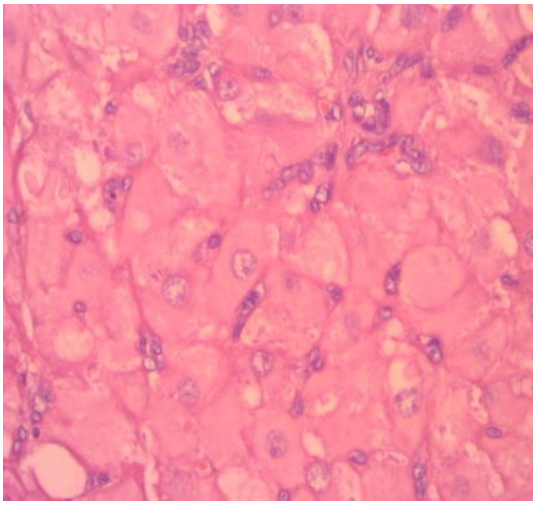
5.4. Cytoplasmic Staining

Regards to cytoplasm staining, H&TM compared to H&E methods pathologist I,II&III reported as 34(85%),38(95%) and 36(90%) conventionally H&E stained slides were adequate and 5,4&5 in adequate respectively. while H&TM method 35(87.5%), 36(%) and 35(87.5%) adequate for cytoplasmic staining by pathologist I, II and III respectively. from 40 stained slides each pathologist evaluated, disagreement reported in 5,4 &5 pairs as reported by pathologist I, II&III respectively. Within comparison method H&T and H&E pathologist I,II &III reported as 20(50%) 22(55%) and 19(47.5%) adequate cytoplasmic staining and 20(50%),18(45%) and 21(52.5%) inadequate cytoplasm stain respectively for H&T method. There was no statistical significance difference among pathologist the chi-square test p value was >0.05.

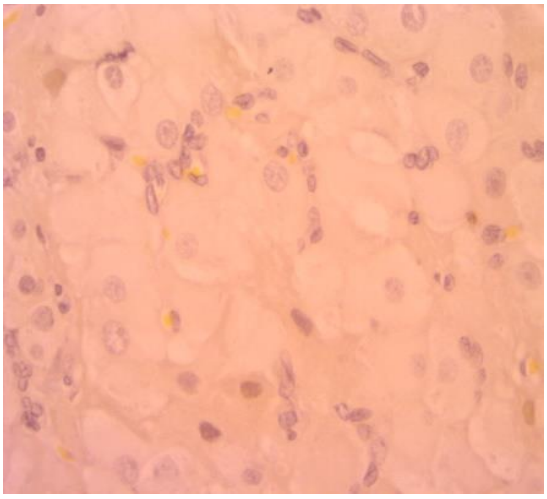
All pathologists favored conventional H&E stained slides over H&TM and H&T stained slides. Both "Z" and chi-square test's p value >0.05 indicated no significant difference b/n the methods of H&TM & H&E, but there was significant difference within H&T method that was chi-square p value was 0.00< 0.05.

Table-4 comparing of cytoplasmic staining of the Hematoxylin&Eosin with Hematoxylin& Turmeric with mordant and Hematoxylin&Turmeric without mordant stained sections.

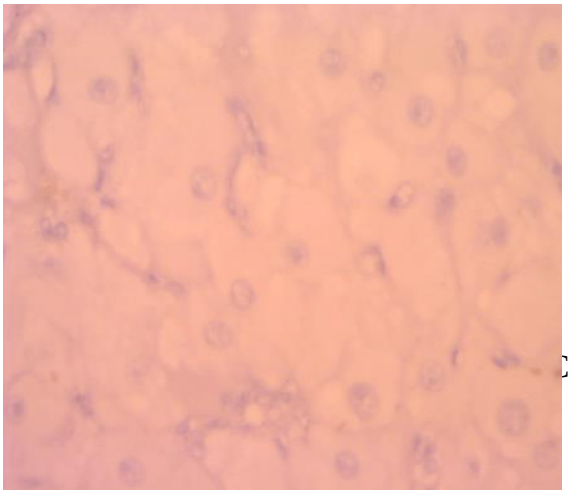
Cytoplasmic staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value (p value)
		inadequate	adequate	total	0.6(0.745)	inadequate	adequate	total	4.1(0.000)
	Inadequate	5	1	6		2	0	2	
	Adequate	0	34	34		18	20	38	
	total	5	35	40		20	20	40	
Pathologist II	Inadequate	2	2	4	0.2 (0.396)	0	0	0	4 (0.000)
	Adequate	0	36	36		18	22	40	
	total	2	38	40		18	22	40	
Pathologist III	Inadequate	4	0	4	0.5(0.723)	2	0	2	4.3 (0.000)
	adequate	1	35	36		19	19	38	
	total	5	35	40		21	19	40	



A. H&E (4X)



B. H&TM (4X)



C. H&T (4X)

Fig.5 **Photomicrograph shows adequate cytoplasmic stain of ovarian tissue within the three methods.**

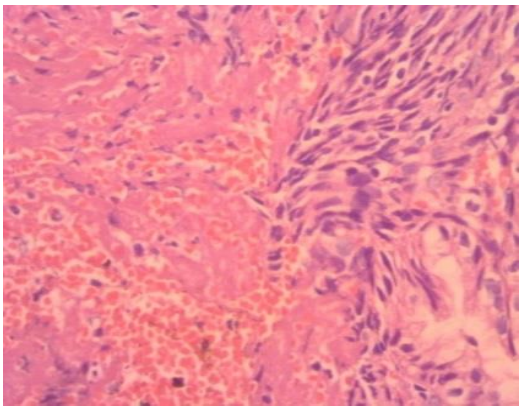
5.5. Uniformity of Staining

pathologist I,II&III reported that uniformity was present 34(85%),38(95%) and 36(90%) of H&E stained slides respectively. while H&TM method 35(87.5%), 36(90%) and 35(87.5%) were presence uniformity respectively. from 40 stained slides each pathologist evaluated, disagreement in 5,4 &5 respectively. Whereas H&T compared to H&E method pathologist I, II &III showed 39(97.5%) ,40(100%) and 40(100%) presence uniformity respectively by H&E . on other hand 33(82.5%),35(87.5%) &29(72.5%) were presence uniformity within the method of H&T, in 7,5,11pairs discrepancy was occurred respectively. there was no statistical significant difference among pathologists the chi-square test p value was >0.05.

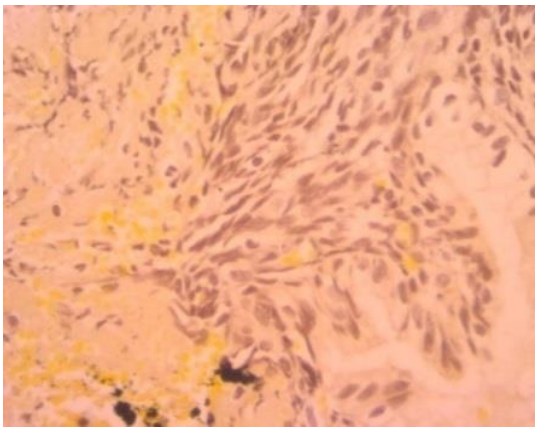
All pathologists favored conventional H&E stained slides over H&TM and H&T stained slides. But there was no statistical significance difference among the two methods conventional and H&TM "Z" and chi-square test's p value was >0.05 (0.150). Significant difference was seen in the method of H&T "Z" and chi-square test's p value was <0.05 (0.00).

Table-5comparing present of staining uniformity of the Hematoxylin & Eosin with Hematoxylin& Turmeric with mordant and Hematoxylin & Turmeric without mordant stained sections

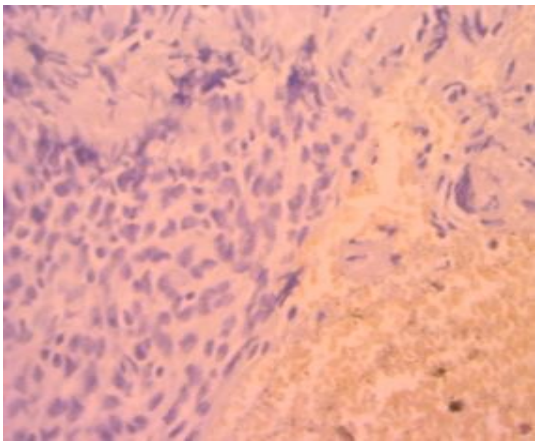
Uniformity of staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		absence	presence	Total	0.3 (0.644)	Absence	Presence	total	1.9 (0.025)
	Absence	2	0	2		1	0	1	
	Presence	1	37	38		6	33	39	
	total	3	37	40		7	33	40	
Pathologist II	Absence	0	0	0	0.5 (0.314)	0	0	0	2.0 (0.021)
	Presence	1	39	40		5	35	40	
	total	1	39	40		5	35	40	
Pathologist III	Absence	0	0	0	1 (0.152)	0	0	0	4 (0.000)
	Presence	2	38	40		11	29	40	
	total	2	38	40		11	29	40	



A H&E (4X)



B. H&TM (4X)



C. H&T (4X)

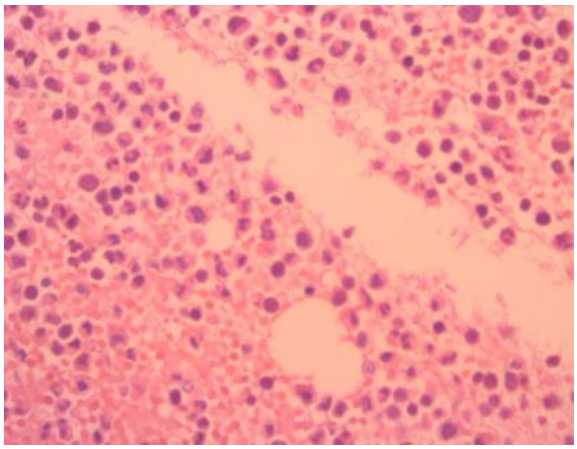
Fig.6 comparison of uniformity endometrial tissue Photomicrographs showing adequately stained

5.6. Clarity of Staining

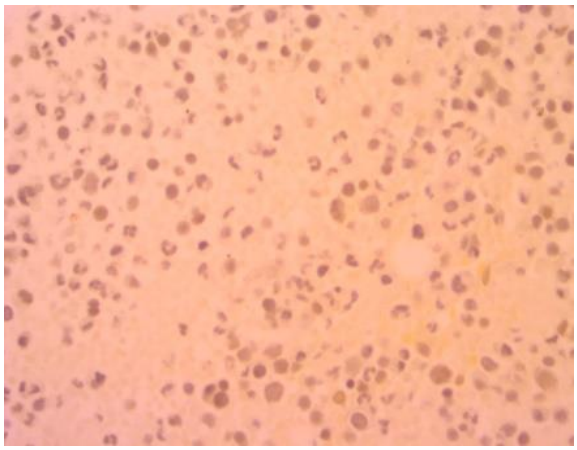
H&TM method was compared with the conventional method H&E for clarity , pathologists (I, &II) reported as 37(92.5%) of conventionally H&E stained slides as clear, and 35(87.5%)& 36(90%) of H&TM stained slides showed clarity respectively. pathologist III revealed that 38(95%)of conventional stained slides and 37(92.5%) of H&TM stained slides were clear. from 40 pairs of stained slides Pathologist I,II& III reported 5,4&3untied pairs respectively. Here there was no statistical significance difference among pathologists, chi-square p value was >0.05 (0.350). Within the method of H&T comparison with that of conventional method 24(60%) and 22(55%) reported clear by pathologist I&III respectively pathologist II, reported that 39(97.5%) clear. From 40 pairs of stained slides, pathologist I and III reported 16 and 18 untied pairs respectively while pathologist II showed no difference between the methods for clarity of H&T staining chi-square p value was > 0.05(0.314). Significant difference between the two methods on clarity is indicated by pathologist I & III. Chi-square p value was <0.05(0.00).

Table-6 comparing the staining of clarity between Hematoxylin & Eosin with Hematoxylin & Turmeric with mordant and Hematoxylin & Turmeric without mordant stained sections

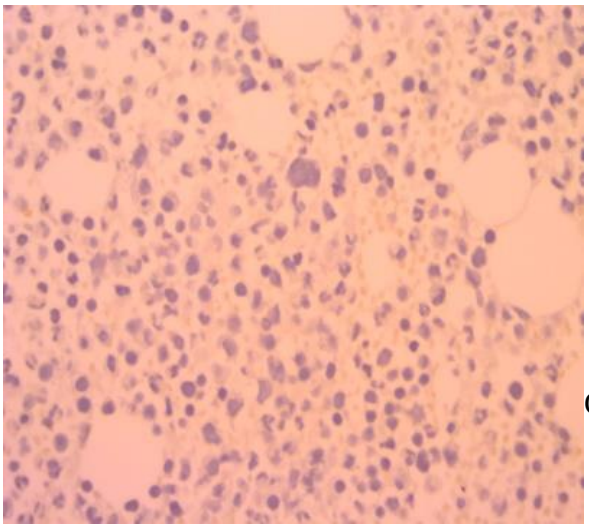
Clarity of staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		Absence	Presence	total		Absence	Presence	total	
	Absence	3	0	3	0.1 (0.456)	1	0	1	4 (0.000)
	presence	2	35	37		15	24	39	
	total	5	35	40		16	24	40	
Pathologist II	Absence	3	0	3	0.5 (0.692)	0	0	0	0.5 (0.314)
	Presence	1	36	37		1	39	40	
	total	4	36	40		1	39	40	
Pathologist III	Absence	2	0	2	0.3 (0.644)	0	0	0	3.9 (0.000)
	Presence	1	37	38		18	22	40	
	total	3	37	40		18	22	40	



A.H&E (4X)



B.H&TM (4X)



C. H&T (4X)

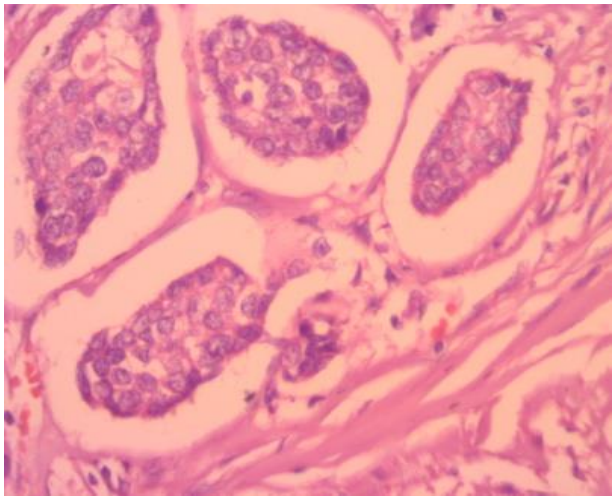
Fig.7 **Photomicrographs showing adequately clear stained bone marrow biopsy with in the three staining methods.**

5.7. Crispness of Staining

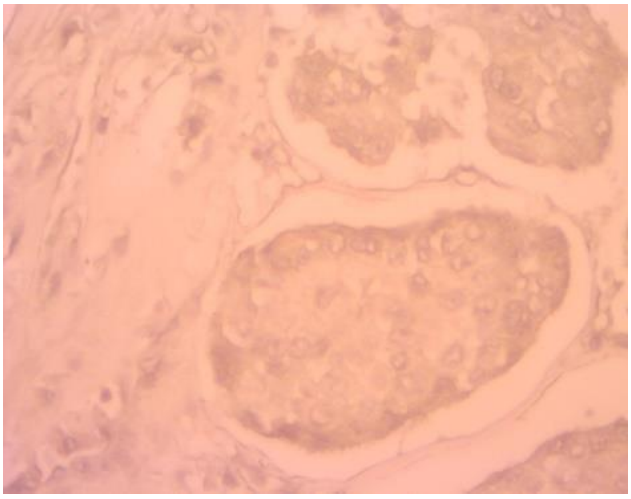
Pathologist I, II&III reported 38(95%), 37(92.5%) and 37(92.5%) conventional H&E stained slides showed crisp respectively. Within the method of H&TM 37(92.5%), 36(90%)and36(90%) were crisp reported by pathologist I,II&II respectively 3,4&4 disagreed. while H&T compared to conventional H&E 15(37.5%),13(32.5%)and15(37.5%) were crisps within the staining of H&T reported pathologist I,II&III respectively. Crispness of staining showed disagreement 25,27&25 pairs stained slides within H&T. there was no statistical significant difference among pathologist chi-square test p value >0.05.All pathologist favored conventional H&E stained sections over H&TM and H&T. Both "Z" and chi-square test p value was>0.05(0.473) no significant difference between the methods of H&TM and H&E, but showed significant difference with H&T chi-square test p value<0.05(0.00).

Table-7 comparing the staining of crispness of the hematoxylin & eosin with hematoxylin & turmeric with mordant and hematoxylin & turmeric without mordant stained sections

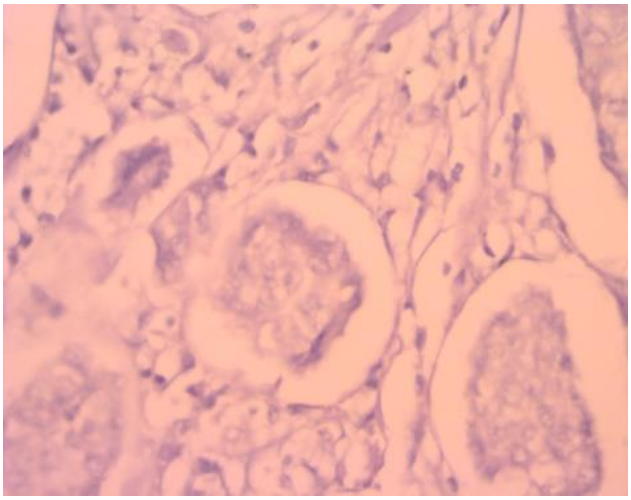
Crispness of staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		Absence	Presence	total		Absence	Presence	total	
	Absence	2	0	2	0.3 (0.644)	2	0	2	3.9 (0.000)
	Presence	1	37	38		23	15	38	
	total	3	37	40		25	15	40	
Pathologist II	Absence	3	0	3	0.5 (0.692)	2	0	2	4.1 (0.000)
	Presence	1	36	37		25	13	38	
	total	4	36	40		27	13	40	
Pathologist III	Absence	3	0	3	0.5 (0.692)	0	0	0	3.0 (0.000)
	Presence	1	36	37		25	15	40	
	total	4	36	40		25	15	40	



A.H&E (4X)



B.H&TM (4X)



C.H&T (4X)

Fig.8 **Breast tissue photomicrograph shows present of crisp within the three methods.**

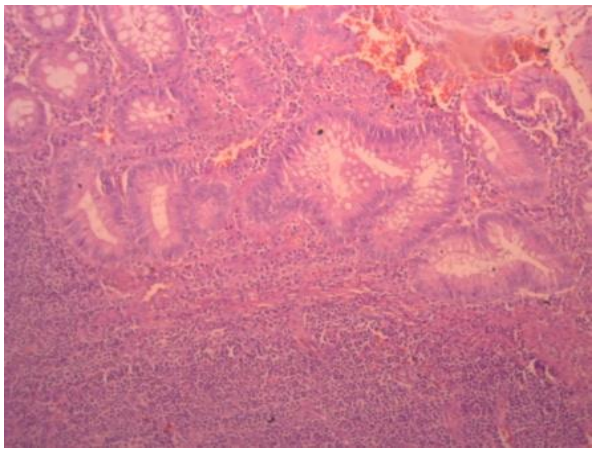
5.8. Background of Staining

Pathologist I, II&III reported 39(97.5(%), 38(95%) &40(100%) conventional H&E stained slides absence of background stain respectively. Within the method of H&TM 39(97.5%), 37(92.5%) and 38 (95%) were absence of background stained reported by pathologist I,II&II respectively 1,3&2 disagreed. while 37(92,5%), absence of background stained within the staining of H&T reported pathologist I,II&III respectively. There was no statistical significant difference among pathologists chi-square p value was > 0.05(0.308)

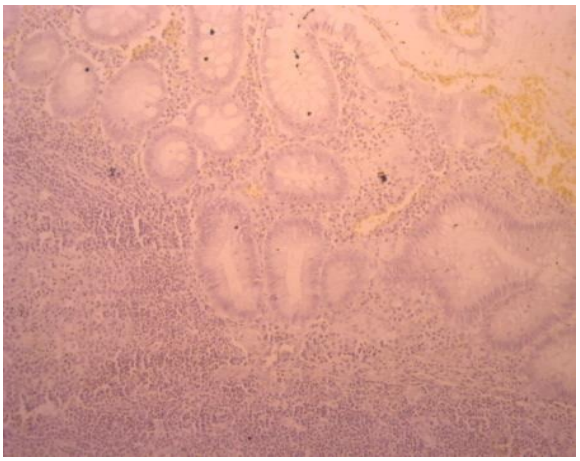
Both "Z" value and chi-square test p value showed no significant difference between the methods of H&E and H&TM, but showed significant difference with H&T p value was< 0.05(0.002).

Table-8 comparing the absence of background staining of the Hematoxylin&Eosin with Hematoxylin& Turmeric with mordant and Hematoxylin&Turmeric without mordant stained sections

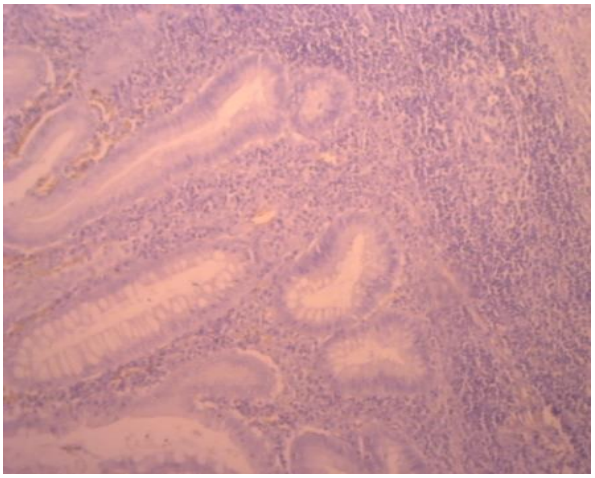
Background of staining									
pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		absence	presence	total		absence	presence	total	
	absence	1	0	1	0 (1.000)	0	0	0	1.4 (0.077)
	presence	0	39	39		3	37	40	
	total	1	39	40		3	37	40	
Pathologist II	absence	2	0	2	0.3 (0.644)	0	0	0	1.4 (0.077)
	presence	1	37	38		3	37	40	
	total	3	37	40		3	37	40	
Pathologist III	absence	0	0	0	1 (0.152)	0	0	0	1.4 (0.077)
	presence	2	38	40		3	37	40	
	total	2	38	40		3	37	40	



A.H&E (4X)



B. H&TM (4X)



C.H&T (4X)

Fig.9 **specimen of appendix photomicrograph shows that the absence of background stains among the three staining methods**

5.9 Adequacy of the three Methods for Diagnosis

H&TM and HE stains were compared for diagnosis and the result showed agreement in 37, 38 &37matched pairs adequate, 3, 2&3 disagreements occurred and comparative result with conventional method with consistent agreement to one another for each parameter. There was no statistical significant difference among pathologist as well as the method chi-square test value was >0.05(0.123). On the other hand adequacy for diagnosis of H&T compared to the conventional the result reported as 22(55%), 24(60%) & 23(57.5%) by pathologist I.II&III respectively and 18,16&17 pairs untied. there was statistical significant difference among methods chi-squared test p value was<0.05(0.00) .

Table- 9 comparing of adequacy of staining for diagnosis by the Hematoxylin & Eosin with Hematoxylin & Turmeric with mordant and Hematoxylin&Turmeric without mordant stained sections.

Adequacy of staining for diagnosis									
Pathologists	Staining methods								
Pathologist I	H&E	H&TM			Z value(P-value)	H&T			Z value(P-value)
		inadequate	adequate	total		inadequate	adequate	total	
	Inadequate	1	0	1	0.5 (0.305)	0	0	0	4.1 (0.000)
	Adequate	2	37	39		18	22	40	
	total	3	37	40		18	22	40	
Pathologist II	Inadequate	1	0	1	0.1 (0.556)	0	0	0	4 (0.000)
	Adequate	1	38	39		16	24	40	
	total	2	38	40		16	24	40	
Pathologist III	Inadequate	1	0	1	0.5 (0.305)	0	0	0	3.0 (0.000)
	adequate	2	37	39		17	23	40	
	total	3	37	40		17	23	40	

6. DISCUSSION

In this study substitution of eosin by a natural dye turmeric for staining biopsy sections and compared with the routine H&E staining procedure. Therefore, the results of each pathologist were analyzed and discussed separately. All results that show significant difference between the methods favor conventional stained sections over H&TM and H&T stained pair. There are some Factors associated with selective staining like dye concentration, time of action on the solvent, its aqueous or alcoholic nature and its ph. chromatography,use of mordant and storage(shelf life) . It was observed that the mode of staining and pH of turmeric stain and eosin were similar. [20, 39] The basic principle in the theory of staining is the ionic bond between the tissue components and the dye, which is associated with electrostatic attraction between the dissimilar ions.

Phytochemical screening of the dye confirmed that presences of main components of turmeric are alkaloids, saponins, flavonoids and tannins. Tannins and flavonoids are responsible for its staining properties (color). Tannins are the most important ingredients which are necessary for dyeing. Saponins are known to reduce surface tension and this property also enhances staining Flavonoids are primarily recognized as the pigments responsible for the autumnal burst of hues and the many shades of yellow, orange and red in flowers and food. 90% of all yellow dyes are flavonoids. Flavonoids typically polyphenolic compounds. Phenols are acidic, due to their ability to release the hydrogen from their hydroxyl group and hence the ability of C. longa to stain the basic parts of the cell, mainly protein part of cytoplasm. [26]

The storage shelf life of turmeric stain is a natural one, it tends to achromatize when stored over a long period of time. The shelf life of turmeric was found to be inferior to eosin. [33]

In the present study turmeric was extracted by maceration method. Addition of mordant and without mordant was compared with the conventional eosin. Comparative study between the two methods was the principal of its kind. The need for mordant in certain histochemical reactions has been anxious. Mordant is a substance that binds the stain on tissue sections by forming a coordination complex and the retention property of the stain to the tissue is enhanced.

Nuclear stain: In this study nuclear staining was evaluated. H&TM result shows that 95.8% adequate nuclear staining when compared to the conventional H&E 99.2%. There was no statistical significance difference among pathologists as well as the methods p-value >0.05. The reason behind could be the addition of acetic acid in the turmeric preparation which decreased the Ph of solution and making it more acidic. This acidic turmeric solution could not stain the basophilic nuclear component of the tissue. Hence it could not stain the nucleus. The second reason might be due to the addition of mordant in turmeric solution has the capacity to enhance nuclear – dye affinity.

On the other hand comparison of H&T with H&E revealed 50.8% and 99.2% adequate nuclear staining respectively. There was no statistical significance difference among pathologists. There was highly significant difference among methods. This could be due to the absence of mordant in turmeric solution utilized.

This result is similar to the study done on kumkum and turmeric where the result indicated100% and 78.95 % adequacy respectively [38].

Cytoplasm stain: Cytoplasm stain of H&TM was compared with that of conventional H&E stain which revealed 88.3% and 96.7% of adequate respectively. Based on this finding there was no statistical significance difference among methods and pathologists p-value>0.05.On the other hand, Comparison showed that H&T with the conventional H&E, 50.8% and 96.7% adequate cytoplasmic stain respectively. There was no statistical significance difference among pathologists p-value. But highly significant difference was seen among methods p-value<0.05.

This difference primarily might be due to the addition of more acetic acid that made the solution more acidic. This leads enhancement of the affinity between cytoplasm (acidophilic) and turmeric solution. Secondly, Preparation of turmeric solution with 70% alcohol increased homogeneity of the solution there by provides more color to the stain. The presence of mordant in the solution was also helped to fasten turmeric stain attachment with cytoplasm. Similar studies carried out on substitution of eosin were able to evaluate their cytoplasm staining character. Their result indicated that cytoplasm staining was better on those with mordant than without mordant. Some other studies also concluded that *Jinger* and *kukum* is better than turmeric but comparable with eosin· [20, 33, 38].

Uniformity and clarity: Another parameter evaluated by pathologists was the presence and absence of uniformity. From the finding it was found that 95.0% H&TM, 80.8% H&T had uniformity as compared to 99.2% of conventional H&E. there was no statistical significant difference among method as well as between pathologists p-value >0.05. The presence or absence of clarity also compared. The result revealed that 90.0% H&TM and 70.8% of H&T were clear when compared to 99.2% of conventional H&E. No statistical significant difference was observed among method as well as pathologists p-value >0.05. The reason behind this result could be due to proper dewaxing, washing and sufficient staining time given during staining.

Crispness: Another parameter evaluated by pathologists was the presence or absence of crispness. From the finding it was found that 90.8% H&TM, 35.8% H&T had crisp as compared to 96.7% of conventional H&E. Statistical significant difference was not shown among pathologists p-value >0.05unlike methods. The difference was between H&T to H&E method of staining. P-value <0.05. Other similar study done on eosin substitution indicated that jinger (50.8%) much better than H&T (35.8%) but very low as compared to H&TM (90.8%) [33].This might be due to the mordant used.

Background of staining: Microscopic stained slides need to have good background stain. In this study background stain was also evaluated. With this point evaluators assessed the presence or absence of back ground staining of slides prepared through H&TM, H&T&H&E which revealed 95.0%, 92.5% &100% good background stain respectively. There was no statistical significance difference among pathologists as well as between the methods. P-value>0.05. The result of H&TM was similar to the reports of other studies (100%) but not with that of H&T .This also might be due to mordant as there was no difference in the two staining methods except for the presence or absence of mordant [33].

Adequacy of diagnosis: on average from a total of 120 stained slides, 112(93.3%) stained slides were adequate for diagnosis and 8 (6.7%) inadequate using H&TM. This revealed that slide stained H&TM were adequate for diagnosis comparably with that of the conventional eosin method appreciable result (p=0.123). There was no statistical significant difference among pathologist. This was supported by study done in 2017 (comparison).The reason behind might be the present of mordant in turmeric solution that binds the dye strongly with cytoplasm, nature of dye (natural dyes need mordant), minimized effect of differentiator and increase shelf life. On the other hand from a total of 120 stained slides 69(57.7%) were adequate for diagnosis and 51(42.5%) inadequate. The study also evaluated and compared the adequacy of HT with that of eosin. In this study there is high discrepancy in adequacy as compared to eosin. There was statistical significant difference among method (p=0.000).This finding was similar to other studies [20, 33]

7. Limitation of the study

Lack of Published paper on turmeric stains on paraffin block tissue Section.

- The gap observed on basic staining characteristics parameters of stain were not checked using different kinds of nuclear or cytoplasmic stains.

8. Conclusion and Recommendation

8.1Conclusion

Evaluation of nuclear, cytoplasm & crispness of staining slides with H&TM was adequate .but not H&T.

Evaluation of uniformity, clarity & background of staining slides with H&TM and H&T adequate.

Evaluation of diagnosis ability stained slides indicated that TM adequate for diagnosis and comparable to conventional H&E the result also help concluded TM has the ability to substitute conventional eosin without compromisation of the result. Whereas T without mordant inadequate for diagnosis lack substituting capacity. Also from economical, safety, availability and comfortable aspect of staining methods substituting eosin with turmeric using mordant has equivalent adequacy for diagnosis. Therefore turmeric with mordant can substitute eosin but not turmeric without mordant.

8.2 Recommendation

FMOH, RHBs shall introduce the use of safe and economical reagent turmeric stain for eosin while expanding pathology service centers throughout the country.

Further research shall be done by agriculture professionals to exactly know the dye content phytochemical of Ethiopian turmeric plants and scholars shall expand the finding by increasing the sample size and catchment area.

I recommend another study by including more pathologists for evaluation with increase the number of sample size and staining approach using turmeric.

9. References

1. Lahiani A, Klaiman E, Grimm O. Enabling histopathological annotations on immunofluorescent images through virtualization of hematoxylin and eosin. Journal of pathology informatics. 2018; 9. https://doi.org/10.4103/jpi.jpi_61_17.
2. Hani A Alturkistani, Faris M Tashkand & Zuhair M Mohammedsaleh A Literature Review and Case Study (2015). Received: May 7, 2015 Accepted: May 31, 2015 Online Published: June 25, 2015 doi:10.5539/ gjhs. v8n3p72 URL: http:/ /dx.doi. org/10.5539/gjhs.v8n3p72
3. Musumeci G. Past, present and future: overview on histology and histopathology. Journal of Histology and Histopathology. 2014; 1(1):5.
4. Agrawal BJ, Influence of mordant application on the dyeing of nylon substrate with natural dyes extracted from flowers:International Research Journal of Chemistry and Chemical Sciences, 2017 4(1): 067-074.
5. SlaouiM,FietteL.Histopathologyprocedures:fromtissuesamplingto histopathological evaluation. in Drug safetyevaluation 2011 (pp. 69-82). Humana Press. https://doi.org/10.1007/978-1-60761-849-2_4.
6. Black JG, Black LJ. Microbiology: principles and explorations. John Wiley & Sons; 2018 Jan4.
7. Fox H. Is H&E morphology coming to an end?. Journal of clinical pathology. 2000 Jan 1; 53(1):38-40.
8. Titford M. A short history of histopathology technique. Journal of Histotechnology. 2006 Jun 1; 29(2):99-110.
9. Busse M, Müller M, Kimm MA, Ferstl S, Allner S, Achterhold K, Herzen J, Pfeiffer F. Three-dimensional virtual histology enabled through cytoplasm-specific X-ray stain for microscopic and nanoscopic computed tomography. Proceedings of the National Academy of Sciences. 2018 Mar 6; 115(10):2293-8.
10. Dapson RW. Benzidine-based dyes: effects of industrial practices, regulations, and world trade on the biological stains market. Biotechnic & Histochemistry. 2009 Jan 1;84(3):95-100.

11. Gupta VK. Fundamentals of natural dyes and its application on textile substrates. Chemistry and technology of natural and synthetic dyes and pigments. 2019 Dec 22:2019.
12. Patil PD, Rao CR, Wasif AI. Revival of natural dyes: Smart use of biodiversity. Colourage. 2012; 10:33-8. Dr. Padma S Vankar.
13. Suvarna KS, Layton C, Bancroft JD. Bancroft's Theory and Practice of Histological Techniques E-Book. Elsevier Health Sciences; 2012 Oct 1. [Accessed date November, 21, 2017]
14. Patel BH. Natural dyes. InHandbook of textile and industrial dyeing 2011 Jan 1 (pp. 395-424). Wood head publishing.
15. Samanta AK, Agarwal P. Application of natural dyes on textiles. Received 18 August 2009; accepted 22September2009at:https://www.researchgate.net/publication/279573040
16. RahmanH.utilization of eosin dye as anion pairing agent for determination of pharmaceuticals: a brief review. Int J Pharm. 2017; 9(12):1-9. Available from: https:// in novare academics. in/journals/index.php/ijpps/article/view/21220
17. Occupational safety and health administration Osha, Material Safety Data Sheet, considered a hazardous substance. 2009-2010
18. . National Center for Biotechnology Information. Pub Chem Compound Summary for CID11048,Eosin.Retrieved June12,2021fromhttps://pubchem.ncbi.nlm.nih.gov/compound/Eosin.
19. Sudhakaran A, Hallikeri K, Babu B. Natural stains Zingiber officinale Roscoe (ginger) and Curcuma longa L. (turmeric)–A substitute to eosin. Ayu. 2018 Oct; 39(4):220.
20. Abraham M, Nambiar S, Charagannavar V, Augustine D, Sowmya SV, Babu A, Rao RS. Comparison of Staining Efficacy between Turmeric and Eosin: A Histological Study. Journal of Clinical & Diagnostic Research. 2017 Nov 1; 11(11).
21. . Kifelew H, Bekele D, Yadesa L, Getu A, Getachew W, Hailemichael G, Mitiku H. Result of turmeric variety trial in Ethiopia. Int. J. Res. Stud. Agric. Sci. 2018; 4(9):34-8.
22. . Mekonnen B, Garedew W. Growth, yield, and quality responses of turmeric (Curcuma longa L.) to nitrogen fertilizer rate and timing of its application. Acta Agrobotanica. 2019; 72(3).

23. Noura S. Dosoky 1 and William N. Setzer:" Chemical Composition and Biological Activities of Essential Oils of Curcuma Species" Received: 4 August 2018; Accepted: 28 August 2018; Published: 1 September 2018
24. Li S, Yuan W, Deng G, Wang P, Yang P, Aggarwal B. Chemical composition and product quality control of turmeric (Curcuma longa L.) 2011; FacultyPublications.Paper1. http://scholarworks.sfasu.edu/agriculture_facultypubs/1
25. Aggarwal ML, Chacko KM, Kuruvilla BT. Systematic and comprehensive investigation of the toxicity of curcuminoid-essential oil complex: A bioavailable turmeric formulation. Molecular Medicine Reports. 2016 Jan 1; 13(1):592-604.
26. Rubina MP, Ashida M Krishnan, Riyas Basheer KB, Mohammed Safeer TK, Soumya V, Assessment of Staining quality of curcumin as substitute for eosin in Hematoxylin &Eosin staining in Histopathology Res Med Dent Sci,2020,8(5):146-150.
27. Labban L. Medicinal and pharmacological properties of Turmeric (Curcuma longa): A review. Int J Pharm Biomed Sci. 2014; 5(1):17-23.
28. Aggarwal ML, Chacko KM, Kuruvilla BT. Systematic and comprehensive investigation of the toxicity of curcuminoid-essential oil complex: A bioavailable turmeric formulation. Molecular Medicine Reports. 2016 Jan 1; 13(1):592-604.
29. Soleimani V, Sahebkar A, Hosseinzadeh H. Turmeric (Curcuma longa) and its major constituent (curcumin) as nontoxic and safe substances. Phototherapy Research. 2018 Jun; 32(6):985-95.
30. Mohandas R, Ramani P, Sherlin HJ, Gheena S, Ramasubramanian A, Don KR, Jayaraj G, Santhanam A. Organic stains used in histopathology-A systematic review. Drug Invention Today. 2019 Feb 1; 11(2).
31. Avwioro OG, Onwuka SK, Moody JO, Agbedahunsi JM, Oduola T, Ekpo OE, Oladele AA. Curcuma longa extract as a histological dye for collagen fibres and red blood cells. Journal of anatomy. 2007 May; 210(5):600-3.
32. Suryawanshi H, Naik R, Kumar P, Gupta R. Curcuma longa extract–Haldi: A safe, eco-friendly natural cytoplasmic stain. Journal of oral and maxillofacial pathology: JOMFP. 2017 Sep; 21(3):340.
33. Sudhakaran A, Hallikeri K, Babu B. Natural stains Zingiber officinale Roscoe (ginger) and Curcuma longa L.(turmeric)–A substitute to eosin. Ayu. 2018 Oct; 39(4):220.

34. Ananthalakshmi Ramamoorthy SR, Jeddy N, Thangavelu R, Janardhanan S. Natural Alternatives for Chemicals Used in Histopathology Lab-A Literature Review. Journal of clinical and diagnostic research: JCDR. 2016 Nov; 10(11):EE01.
35. . Kumar S, Singh NN, Singh A, Singh N, Sinha RK. Uses of Curcuma longa L. extract to stain various tissue samples for histological studies. Ayu. 2014 Oct; 35(4):447.
36. Bassey RB, Oremosu AA, Osinubi AAA. Curcuma Longa: Staining Effect on Histomorphology of the Testes. Maced J Med Sci. 2012 Mar 15; 5(1):26-29. http://dx.doi.org/10.3889/ MJMS.1957-5773.2011.
37. Bondoc CC. Curcuma longa Linn rhizome extract as an alternative stain for histological studies. Journal of Pharmacognosy and Phytochemistry. 2018; 7(5):3010-7.
38. NAVYA N, KUMARGURU B, RAMASWAMY A, SWETHADRI G, JANAKIRAMAN P. Utility of Kumkum as a Counterstain in Histopathological Evaluation of Cervix: An Unexplored Archaic Behooveful Colourant. Journal of Clinical & Diagnostic Research. 2021 Apr 1; 15(4).
39. Kurien BT, Dorri Y, Scofield RH. Curcumin/Turmeric as an Environment-Friendly Protein Gel Stain. InProtein Gel Detection and Imaging 2018 (pp. 121-131). Humana Press, New York, NY.
40. Kamsu Tchuente G, Fodouop Chegaing SP, Tagne RS, Kodjio N, Fakam Nguelebeck AL, Gatsing D (2019) Evaluation of the acute and sub-chronic toxicity of the ethanolic extract of Curcuma longa (Zingiberaceae) in Wistar albino rats. Mod Chem Appl 7:267. doi: 10.35248/2329-6798.19.7.267.

10. Annex

Annex –1: staining solution preparation procedure

A. H&E Stain

Hematoxylin……2.5gm
Absolute alcohol……25ml
Potassium alum……50gm
Distilled water……..500ml
Sodium iodate……0.5gm
Glacial acetic acid……...10ml

0.3% Eosin Y solution

Eosin Y……3gm
Distilled water…….1000ml

0.3% Acid alcohol

HCL……3ml
70% Alcohol…….997ml

0.5% Lithium carbonate

Lithium carbonate…….5gm
Distilled water……1000ml

B. H&TM Stain

Harris hematoxylin similar to H&E

0.3% Acid alcohol similar to H&E

0.5% Lithium carbonate (similar to H&E)

Turmeric powder…….15g.

70% ethanol (prepared from absolute ethanol 99.9% and distilled water)……100ml.

Potassium aluminum sulfate alum mordant…….17g

Glacial acetic acid……4ml

C. H&T Stain

Harris hematoxylin similar to H&E

0.3% Acid alcohol similar to H&E

0.5% Lithium carbonate (similar to H Turmeric powder…….15g.

70% ethanol (prepared from absolute ethanol 99.9% and distilled water)…….100ml

Glacial acetic acid……4ml

Annex-2: processing reagents and schedule

No.	reagents	Schedule time set.	Temperature
1.	4% formalin	1 hour	35
2.	water	5 seconds	35
3.	50% ethanol	1hour	35
4	70% ethanol	1hour	35
5.	80%ethanol	1hour	35
6.	90% ethanol	1hour	35
7.	100% ethanol	1hour	35
8.	100%ethanol	1hour	35
9.	xylene	1hour	35
10.	xylene	1hour	35
11	Paraffin wax	45 minutes	68
12.	Paraffin wax	45minutes	68
13	Paraffin wax	45minutes	68
14.	Paraffin wax	45minutes	68

Annex-3: Protocols of the three stains used in the Study

I. Hematoxylin and Eosin Stain

Staining procedure:

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Wash in distilled water for 10 min
3. Stain with Harris's hematoxylin stain for 7 min.
4. Wash slides in tap water and differentiate in 1% acid alcohol
5. Wash in tap water for 10 min and dip slides in lithium carbonate for bluing for 5 min
6. Stain slides with eosin for 15 sec.
7. Slides will be dehydrated with graded alcohol, cleared in xylene and mounted.

Results

Nuclei……deepblue-purple

Cytoplasm……Eosinophilic(redtopink)

Collagen……Orange to light pink

Redbloodcells……lightpink

Collagen……..Orange-red

Reference : Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. 2008 May 1;2008:pdb.prot4986. doi: 10.1101/pdb.prot4986. PMID: 21356829.

II. Hematoxylin &Turmeric with Mordant Stain

Fixation: 10% NBF

Sections: 4 microns

Solutions used:

Harris hematoxylin

Turmeric with mordant solution

Staining procedure:

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Wash in distilled water for 10 min
3. Stain with Harris's hematoxylin stain for 7 min.

4. Wash slides in tap water and differentiate in 1% acid alcohol

5. Wash in tap water for 10 min and dip slides in lithium carbonate for bluing for 5 min

6. Stain slides with turmeric for 10 min.

7. Slides will be dehydrated with graded alcohol, cleared in xylene and mounted.

Result

Nuclei……blue

Cytoplasm……yellow

Collagen…… deep yellowish orange

Red blood cells…… deep yellowish orange

Reference: Kumar S, Singh NN, Singh A, Singh N, Sinha RK. Uses of Curcuma longa L. extract to stain various tissue samples for histological studies. Ayu 2014; 35:447-51.

III. Hematoxylin &Turmeric without Mordant Stain

Fixation: 10% NBF

Sections: 4 microns

Solutions used:

- Harris hematoxylin
- Turmeric without mordant solution

Staining procedure:

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.

2. Wash in distilled water for 10 min

3. Stain with Harris's hematoxylin stain for 7 min.

4. Wash slides in tap water and differentiate in 1% acid alcohol

5. Wash in tap water for 10 min and dip slides in lithium carbonate for bluing for 5 min

6. Stain slides with turmeric for 10 min.

7. Slides will be dehydrated with graded alcohol, cleared in xylene and mounted.

Result

Nuclei……Blue

Cytoplasm……yellow

Collagen……. deep yellowish orange color

Red blood cells…… deep yellowish orange

Annex-4: Pathologist Checklist

Addis Ababa University, school of medicine post graduate program department of pathology: concept note

This check list prepared for pathologists only for research purpose, the researcher (PI) Belaynesh Zewdu MSc candidate of Addis Ababa University School of medicine department of pathology, trying to evaluate the efficacy of turmeric solution for cytoplasmic stain for histopathological tissues. True and direct information is need while filling the check list and your participation is highly valuable to address the issue. If there any questions or problems encounter during this study contact me by the following options.

Belaynesh Zewdu, mobile number 0912841169.

Email address, zewdubelaynesh@g mail.com

Stained section grading parameters:

- Nuclear staining (inadequate = score 0, adequate = score 1,)
- Cytoplasmic staining (inadequate = score 0, adequate = score 1)
- Uniformity of staining (absent = score 0, present = score 1)
- Clarity of staining (absent = score 0, present = score 1)
- Crispness of staining (absent = score 0, present = score 1).
- Background of staining (absent=score 0, present=score 1)

Slide label/ code/	Nuclear staining	Cytoplasmic staining	Uniformity of staining	Clarity of staining	Crispness of staining	Background of staining

Annex -5: Investigator Check list

Biopsy No.	Recode conventional H&E stain	Recode H&TM stain	Recode H&T stain

ANNEX 6: Declaration

This thesis is as a partial fulfillment of the requirements for the degree of Master of Science from Addis Ababa University, I hereby grant to Addis Ababa University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter known, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Belaynesh Zewdu: _____ Date: _____

Advisor:

Mulugeta Temesgen MD, Associate professor.

Department of Pathology

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