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Review Article

Pathology Section

Natural Alternatives for Chemicals Used in Histopathology Lab- A Literature Review

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ABSTRACT

Histopathology lab is the place where the specimen gets processed and stained to view under microscope for interpretation. Exposure to the chemicals used in these processes cause various health hazards to the laboratory technicians, pathologists, and scientists working in the laboratory. Hence, there is a dire need to introduce healthy and bio-friendly alternatives in the field. This literature review explores the natural products and their efficiency to be used as alternatives for chemicals in the histopathology lab.

Keywords: Natural products, Occupational hazards, Pathology

INTRODUCTION

Histopathology is the study of biologic tissues using a microscope to appreciate the diseased cells. Fixation, dehydration, clearing, embedding, sectioning and staining are the processes involved in converting un-stained tissues to stained sections. Exposure to the chemicals used in these processes can cause various health hazards to the laboratory technicians, pathologists and scientists who frequently get exposed. The causes of death of pathology technicians in United Kingdom were analysed by Harrington and his colleagues from 1950s until the late 1980s [1-3]. It was witnessed that suicide and malignancies were the commonest cause for death. From the above observation it was hypothesized that exposure to formaldehyde could be the aetiology. It is also stated that formaldehyde as a known carcinogen is also an allergen. Literature reveals that the persons who work with toluene and xylene are at increased risk of developing a vascular condition known as Raynaud's phenomenon. The chance of developing severe Raynaud's phenomenon increases by a factor of nine for those who work with toluene and xylene combined with acetone or chlorinated solvents [4]. In the quest to eliminate the use of these toxic chemicals from the laboratory, numerous natural alternatives are being used. This review will be a pilot effort in exploring the natural products and their efficiency to be used as alternatives to chemicals used in the histopathology lab.

Common chemicals used in histopathology lab: Four major groups of fixatives are used namely the aldehydes, oxidizing agents, alcohol based fixatives and the metallic group of fixatives [5]. Formalin is the universal fixative in routine histopathology. Besides its good fixing properties, it has many disadvantages also. The International Agency for Research on Cancer (IARC) classifies formaldehyde as a human carcinogen that can cause nasopharyngeal cancer [6]. Lu et al., found strong evidence that can support a genotoxic and also cytotoxic mode of action for the carcinogenesis of inhaled formaldehyde in respiratory nasal epithelium [7].

Common chemicals used for dehydration are Ethanol, Industrial methylated spirit (denatured alcohol), Methanol, Propan-2-ol, Isopropyl alcohol, Butyl alcohol, Acetone, Universal solvents-Dioxane, Tertiarybutanol, Tetrahydrofuran. Common chemicals used as clearing agents are Xylene, Toluene, Chloroform, Methyl benzoate and methyl salicylate [8]. Xylene which has a good compatibility with alcohol and paraffin wax has been widely used for the past many years as a clearing agent.

The tissue sections, without staining appear colourless and different structures cannot be appreciated. Staining the tissue sections by different coloured dyes, having affinities towards certain components of the tissues, makes identification easy and also helps in study of their morphology. In the past, the readily available chemicals were used by the histologists to prepare tissues for microscopic studies. Potassium dichromate, alcohol and the mercuric chloride were used to process the cellular tissues followed by staining with Carmine, Silver nitrate, Giemsa, Trichrome Stains, Gram Stain and Haematoxylin. Other stains used routinely are Masson's Stain used in connective tissues; Golgi Stain used in neuronal fibres; Toluidine Blue, Immunological labelling that have fluorescent or enzymatic stains, Kluver-Barrera Stain used in Lipofuscin, Periodic Acid-Schiff (PAS) stain, Mallory's CT Stain used in carbohydrates [9]. Advanced stains include: Immunohistochemistry (IHC) and the in situ hybridization. The most commonly used stain for histological study is Haemotoxylin and Eosin (H&E stain).

Effects of commonly used chemicals: It is a fact that, the chemicals in the histopathology lab are human irritants when it goes beyond the threshold level of the tissue. They can cause reversible inflammation to skin, eyes and respiratory passages. Chemicals like chloroform, potassium dichromate, dioxane, formaldehyde, nickel chloride and chromic acid and dyes such as auramine O, basic fuchsin and Congo red are carcinogenic in nature. Certain chemicals are capable of causing death at certain specified concentrations by ingestion, skin contact or inhalation. When these chemical effects have to be avoided in the histopathology lab, then the possibility of alternative agents must be examined. Many of the commonly used organic solvents like ethyl alcohol, methyl alcohol, acetone and diethyl ether have flash points below 21°C and are highly flammable. Though picric acid, an explosive is used in the histopathology lab, other explosive chemicals are otherwise uncommon. Certain silver solutions may explode when in contact with suitable substances. For example, silver nitrate when in contact with ethanol presents a serious fire risk [10]. Considering the serious adverse effects of these chemicals, many attempts have been made to replace them with safer natural alternatives.

Since, time immemorial natural products have been in use for tissue preservation and processing. In history, the ancient Egyptian civilization used natural products as mummification materials such as Natron salt, Myrrh, Beeswax, Bitumen, Cassia, Onions, Lichen, Coniferous resin, Mastic, Henna and Gum Arabic which effectively helped in the preservation of the dead body [11]. Various oils are used as a natural preservative in Indian cookery. Various natural alternatives which can be used as a substitute to the chemicals used in the histopathology lab are discussed below.

Natural Alternatives Used for Fixation

(a) Honey: Honey is essentially a concentrated water solution of two sugars, dextrose and levulose, with 22 other more complex sugars in small amounts. Honey is produced from the floral sources and contains several minerals, vitamins, carbohydrates, ascorbic acid hydrogen peroxide and trace elements. The anti-bacterial property of honey is due to the presence of these components. The presence of acid accounts for the low pH (3 to 4) of honey. Usually, the fixatives that have low pH do not favour the preservation of cytoplasmic organelles; however, they act as good nuclear fixatives. For several centuries, honey has been documented to possess anti-bacterial, acidic and dehydrative properties. The anti-autolysis and tissue hardening property of the honey, besides its wound healing and anti-bacterial nature was highlighted in one of the studies [12]. These are the properties of a fixative that present honey in terms of a fixative rather than a preservative. Vidushi Lalwani et al., evaluated the fixative properties of processed and unprocessed honey when compared with formalin in oral tissues [13]. Fixing and staining efficiency of processed honey and unprocessed honey was at par with neutral buffered formalin with regard to the staining efficacy. Staining efficacy of nucleus, cytoplasm and appreciation of tissue morphology was 100%, 92%, 75% respectively. Their results suggest that, it is safe to use both processed honey and unprocessed honey as an alternative for formalin [13]. Amita Singh et al., analysed the efficacy of cytological smears fixed using ethanol and 20% unprocessed honey. They also compared the efficacy between the two fixatives. The results showed that 90% of the ethanol-fixed smears were sufficiently fixed as compared with the honey-fixed smears, which were 80% adequate. They concluded that both Ethanol-fixed and Honey-fixed smears were at par with each other, and honey could be safely used as a substitute to ethanol [14]. B. Sabarinath et al., did a study with honey that aimed to determine the effectiveness of honey as a fixative when compared with formalin. The results of the study showed that the nuclear details both in honey and formalin fixed specimens were similar with no difference in staining and microscopic morphology. However, the stained cytoplasm was sufficient enough to make out the integrity of the tissue. The cytoplasm of epithelial cells did not undergo any change whereas; the connective tissue cytoplasm exhibited good staining with H&E, with complete homogenization effect on the collagen fibers [12].

(b) Sugar syrup and Jaggery: Sugars, often called culinary sugars (used in cooking) consumed all over the world are manufactured either from sugarcane (70%) or sugar beet (30%) [15]. Jaggery is a traditional Indian sweetener, produced in addition to sugar from sugarcane. It is a natural mixture of sugar and molasses. A solid material (usually possessing sucrose 65-85%) is left when pure clarified sugarcane juice is boiled and is called as jaggery [16].

To evaluate the fixative property of sugar, several studies have been conducted. Patil S et al., studied the tissue fixation property of 20% honey, 20% sugar syrup & 30% jaggery syrup in comparison with 10% buffered formalin using H&E stain. They found that jaggery fixation excelled and the tissue sections had good overall morphology, nuclear, cytoplasmic details and staining quality with clearly discernible cellular outline [17]. Patil S et al., in their another study evaluated the fixative property of 30% jaggery and 20% honey with 10% buffered formalin as control over 6 months and ascertained the results using haematoxylin and eosin (H & E) stain. They also evaluated the compatibility of jaggery and honey fixed samples for special stains such as Periodic Acid Schiff (PAS) and Masson–Trichrome (MT). At the end of 6th month, all the three fixatives demonstrated similar results, with jaggery fixed tissues being comparable to formalin fixed tissues in all the three (H & E, PAS, MT) stained sections [18].

Natural Alternatives Used for Dehydration

The procedures such as freeze-drying and freeze-substitution are found to be a safer alternative for dehydration. These cryo-techniques date back to the pioneering works of R Altmann (1890) and I Gersh (1932) to avoid alterations seen in the use of chemical fixatives and dehydration. Since then, freeze-drying and freeze-substitution techniques are being used in light and electron microscopy experiments. Nevertheless for the disadvantage of its sophistication it is employed only for distinct studies in research laboratories and not in routine histopathology lab [19].

Natural alternatives used for clearing: A solution can be considered as a clearing agent, if it rapidly penetrates into tissues to clear them. The viscosity of the solution plays an important role in easy penetration into tissues. For example, a less viscous solution penetrates faster to that of high viscous solutions. Many essential oils are used as a substitute to the chemical clearing agents such us xylene which is commonly used. These essential oils are used as natural flavouring agents for food, as fragrances in perfume, and in medicine as well as in alternative medicines such as aromatherapy. Clearing agent should be fully miscible with ethanol as well as paraffin wax. This solvent will displace the ethanol, then, this in turn will be displaced by molten paraffin wax [20]. An essential oil, also known as volatile or ethereal oil is a liquid that is distilled mostly by steam or water from different parts of the plant such as leaves, stems, flowers, bark, and roots. Some of these oils used in literature as an alternative for clearing are discussed below.

(a) Coconut oil: Coconut oil or copra oil, is extracted from the kernel or meat of the mature coconuts obtained from the coconut palm (*Cocosnucifera*). It slowly oxidizes because of its high saturated fat content, and is thus, resistant to rancidification. Coconut oil is a commonly used vegetable oil, available all over the tropical world. It is non-toxic and heat stable. Wajid Sermadi et al., in their study evaluated the efficacy of coconut oil as a clearing agent compared to xylene. Results showed that there was no difference seen in staining quality and tissue architecture in both the specimens. They concluded that coconut oil may be substituted for the xylene without compromising the quality of histological details [21].

(b) Cedarwood oil: Cedar wood oil is perhaps the most well known natural wood oil for clearing tissues. It is obtained from juniper and cypress species, both of which are within the general descriptions of being cedar trees. For histological processing a low viscous variety is needed. The viscosity of the oil determine the clearing time. Though the oil takes longer time to process, surprisingly it causes no damage to the tissues. Sudip Indu et al., compared the efficacy of cedarwood oil and xylene in H&E staining procedures. They observed adequate nuclear staining in 90% of sections cleared using cederwood oil and 93.33% with xylene. It was also noticed that cedarwood oil and xylene had alike cytoplasmic staining (93.33%). These qualities makes cedarwood oil to be considered as an uncompromised alternative to xylene as a clearing agent [22].

(c) Bleached palm oil: Mfoniso Udonkang et al., did a study on bleached palm oil as substitute for xylene in histology. The results showed minimal differences between the tissues cleared and de-waxed with bleached palm oil at 60^0c and the xylene counterparts in terms of transparency (93.3%), production of serial sections (73.7%) and quality of histological staining (88.9%). They concluded that it is a safe, economical and locally produced substitute for xylene [23].

(d) Other oils used: BR Premalatha et al., conducted a study which aimed to evaluate the efficacy of mineral oil as a deparaffinising agent when compared to xylene using H & E staining. The study 100% and 93.3% of sections cleared using xylene and mineral oil respectively were adequate for diagnosis. These scientific documentations justify refined mineral oil as a potent natural alternative to xylene [24]. Sugunakar Raju Godishala Swamy et al., recently used carrot oil, olive oil, pine oil and rose oil in their study as an efficient substitute to xylene and showed that all the

four oils had the ability to clear the tissues similar to xylene. Among the oils that were studied, pine oil stands good with the physical and chemical properties of xylene. The stability and longitivity of H&E staining was also evaluated for over a period of one year and no significant difference in staining quality was observed [20]. In contrast, Andrea et al., substituted xylene with a mixture of four oils- peanut oil, soyabean oil, coconut oil and cotton oil and noticed that, it was a poor alternative, as the quality of sections with respect to xylene specimens were better, when compared with natural oils [21].

Lemon water: Anuradha Ananthaneni et al., assessed the efficacy of diluted lemon water (95%) along with 1.5% dish washing solution as deparaffinising agents compared with xylene. Adequacy of nuclear staining, crispness, and staining for the specimens cleared with 95% lemon water, were found to be 100% for diagnosis, whereas the cytoplasmic staining and clarity were observed in 55% and wax retention was seen in 50% [25].

Natural Alternatives Used for Staining

The materials and the technology used in the histopathology lab have been undergoing transformation [9]. Historical staining techniques by early pathologists and surgeons were borrowed from a 17th century scientist Leeuwenhoek, who used substances such as madder, indigo and saffron to stain tissues and using rudimentary microscopes to study them [26]. Haematoxylin (*Haematoxylon campechianum*) is a stain which is commonly used in histopathology lab is also derived from natural source.

Carmine: It is a scarlet dye obtained from the ground bodies of cochineal beetles. It is a commonly used stain in histology used by early botanists such as John Hill in their studies in 1770s [9]. The stain was used to study microscopic tissue structures when in ammonical solution form and it is still used today in histologic studies. In particular, the stain was used widely by Rudolph Virchow [27].

Carcade (*Hibiscus sabdariffa*): *H. sabdariffa* belongs to Malvaceae family, which is a true Roselle plant, a very important dye-yielding annual/perennial plant variety. The aqueous extract of *H.Sabdariffa* is acidic and red in colour. The plant has many industrial, medical, and nutritional uses [28,29]. Ihuma et al., investigated the potential of methanolic extracts from *H. Sabdariffa* for staining fungal species. *Apergillus niger, Rhizopus stolonifer* and *Penicillium notatum* were stained with methanolic extracts from *H. Sabdariffa* and compared microscopically with Lactophenol-in-cotton blue. *H. Sabdariffa* preparations appeared more contrasted as compared to Lactophenol-in-cotton blue stained preparations. So they concluded that methanolic extracts from *H. sabdariffa* could be used as a mycological stain [28]. Raheem et al., attempted to document the staining efficiency of *H. sabdariffa* on skin tissue compared with routine H&E stain. Best results were obtained when 5% solution was used for 60 minutes. So they concluded that it could serve as a cytoplasmic stain instead of eosin in the H&E method to stain formalin-fixed paraffin-embedded skin tissue sections [29].

Henna (*Lawsonia inermis*): Henna that belongs to the Lythraceae family is a small shrub used as a cosmetic for colouring hair and decorating palms of the women (called as 'Mehandi' in India). Henna gives a red orange dye molecule, "lawsone" (2-hydroxy-1, 4-napthaquinone), which is known as hennotannic acid, that has affinity for bonding with protein and also have a fast-dyeing property. This has been used to dye skin, fingernails, hair, leather, silk and wool [30,31]. Hafiz H et al., studied the potentials of henna leaves extract as counter stain in gram staining reaction. The research recommend that henna leaves extracts oxidised with potassium permanganate can replace the counter stains in gram staining reaction [30].

Wing fruit (*Pterocarpus osun*): It is a forest tree that comes under the family of papilionaceae and gives off a red pigment. Avwioro et al., determined the staining potential of *Pterocarpus osun* extract on tissue sections. The red pigment was extracted from the powdered stem, with 1L of 70% ethanol at 78°C for 24 hour. The collagen fibres, red blood cells and muscles were stained using the alcoholic, alkaline and acidic extracts which are again hazardous to the environment. They concluded that *P. osun* extract is a promising histological stain that can be used for histopathological diagnosis of diseases [32].

China rose (*Hibiscus rosa sinensis*), Sugar beet/Red beet (*Beta vulgaris*), Red rose (*Rosa hybrida*): *Hibiscus rosa sinensisis* (Malvacea family), is used as a colorant to various edible and in-edible items. Red beet is a member of Chenopadae family. They can be used as a natural colorant in beverages, ice creams and some fruit products. Genus Rosa, belongs to Rosaceae family. It has colourful flowers ranging from white, yellows and reds. Roses are ornamental plants grown for their flowers in the garden through the world [33,34].

Niranjan Kumar et al., studied the staining of platyhelminthes using aqueous and alcoholic extracts of China rose (*Hibiscus rosasinensis*),sugar beet (*Beta vulgaris*) and red rose (*Rosa hybrida*). They have concluded that the extract of roses i.e., red rose followed by China rose, followed by red beet possess the ability to replace the conventional stains in the taxonomic study of Platyhelminthes parasites [33]. Chew Weng Cheng et al., did alternative staining using distilled water and ethanol extracts of hibiscus (*Hibiscus rosa sinensis*) and red beet (*Betavulgaris* L.) against Lugol's iodine in wet mount procedure to diagnose ova of

Laboratory Procedure	Natural Alternatives	Pros	Cons
Fixation	i) Honey	Anti-autolysis and tissue hardening property.	Diluted honey has to be mixed with anti-fungals.
	ii) Jaggery	Cytoprotective and anti-oxidant property.	Altered cross binding with the tissue as compared to formalin.
Clearing	i) Coconut oil	Profusely available in the tropical world, less expensive and non-hazardous.	Tendency to get solidified at a lower Temperature.
	ii) Cedarwood oil	Non-hazardous.	Expensive than other commonly used alternatives.
	iii) Bleached palm oil	Refractive index closer to tissue proteins, infiltrates the intercellular spaces	Doesn't dissolve tissue fats as xylene does
	iv) Refined mineral oil	Paraffin wax can be dissolved in mineral oil completely. Density is closer to that of average density of human fat, allowing easy removal of tissue fat.	Intensity and uniformity of staining are less.
	v) Lemon water	Dissolve wax.	Less uniformity in staining.
Staining	i) Carmine	Very clean and sharp nuclear staining.	Quality is affected by the temperature and degree of illumination during its preparation.
	ii) Carcade	Comparable to eosin in skin section.	More studies are recommended.
	iii) Henna	Wide availability	Need oxidation with potassium permanganate.
	iv) Wing fruit	Good staining potential.	Needs preparation of alcoholic, alkaline and acidic extracts which are hazardous.
	v) China rose	Easily available in nature	Extraction and purification of the stain is a tedious process.
	vi) Sugar beet		
	vii) Red rose		

[Table/Fig-1]: Advantages and Disadvantages of the natural alternatives.

intestinal nematodes (*Trichuris trichiura* and *Ascaris lumbricoides*). Distilled water extraction was found to be the poorest staining dye. Finally, it has been concluded that 50% ethanolic extraction of hibiscus can be applied as a stain in parasitology [34].

The summary as well as of pros and cons of all these natural alternatives have been tabulated in [Table/Fig-1].

CONCLUSION

To go organic is the theme of the present day in order to combat the global warming. Implementing bio-compatible substitutes in routine histopathology is necessary. Though natural products are cost effective and non-hazardous, the efficiency and commercial availability of chemical products makes them indispensable. Furthermore, studies should be made in an aim to explore more natural products with fixing, clearing, dehydrating and staining properties. The authors of this article are currently in an on-going project that evaluates the effectiveness of natural oils as an alternative to chemicals used in the histopathology lab. By combining the natural products, the chemical composition may be modified and new formulae may be derived.

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