Natural fixatives alternative to formalin in histopathology: A systematic review

Adrinna Yee Weng Lum, BMedSc[1], Phyu Synn Oo, PhD[2], Saint Nway Aye, MMedSc[2], Lim Wei-Jet, BMedSc[1], Valerie Chee Chia Xian, BMedSc[1], Purushotham Krishnappa, MD[2]

[1]Biomedical Science Program, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia, [2]Pathology Department, School of Medicine, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia

ABSTRACT

Introduction: Since constant long-term exposure to formaldehyde endangers the health of laboratory personnel, sugar-based natural products have become interesting alternative fixatives to formaldehyde because of their preservative and antibacterial properties. However, there are controversial findings on the fixative effects of natural fixatives. This study systematically reviews the evidence comparing natural fixatives' types, dilutions, fixative properties and staining quality in normal tissues and histopathological specimens.

Materials and methods: A comprehensive search was performed for studies comparing the natural fixatives- and formaldehyde-fixed tissues using databases from inception to January 2022: PubMed, Ovid Medline and Google Scholar. Two independent reviewers did data extraction. The data were pooled for the type of natural fixatives, their concentrations and fixative qualities compared to formaldehyde.

Results: Fifteen studies were included in this systematic review. Nine studies used one natural fixative with different dilutions, while six used several natural fixatives to compare their fixative properties with formaldehyde. The most used natural fixative was honey (n = 12) followed by jaggery (n = 8), sugar (n = 3) and others (n = 1). Honey showed the most promising results in fixation and staining, which are compatible with formalin. Jaggery and sugar also showed the possibility of replacing formaldehyde in tissue fixation and staining in smaller tissue samples.

Conclusion: Natural fixatives showed promising results in tissue fixation. However, optimising the concentrations and conditions of natural fixatives is difficult because of the different chemical constituents and production steps. More comprehensive studies are necessary for application.

KEYWORDS:
Natural fixatives; sugar-based fixatives; honey fixatives; histopathological practice

INTRODUCTION

Fixation is a crucial step in any histopathology setting. Fixation allows tissue sections to be studied microscopically by preserving tissues and preventing bacterial putrefaction or autolysis.[1] Discovered in the 18th century, formaldehyde is used as a gold standard fixative in routine histopathology, with excellent preservative properties. Antiseptic and antiperspirant features were also observed in formalin, encouraging its use in anatomical and histological settings.[2] As formalin is cost-effective and highly efficient, there is hardly a need to seek an alternative to the 'gold-standard' fixative.[3] Despite its benefits, formalin has been known for many years as a potent irritant of the skin and nasal cavity, and it is cytotoxic at high doses. It is considered a carcinogen for nasopharyngeal,[4] and lymphatic and haematopoietic cancers, including leukaemia.[5] Due to its hazardous nature, numerous natural fixatives have been studied to seek an alternative to formalin fixatives. The Occupational Safety and Health Administration (OSHA) has greatly encouraged ongoing research to look for a safer and eco-friendly replacement to formalin fixatives.[6]

Natural products such as honey, sugar and jaggery have preservative properties.[7] Natural fixatives are low-cost, non-toxic and eco-friendly, making them suitable for routine laboratory usage.[6,8] Honey has antibacterial and dehydrating properties.[9-11] Anti-autolysis and tissue hardening qualities were also highlighted in the use of honey.[9] In addition, scientific studies have proven that natural fixatives can preserve tissue morphology similarly to formalin with no interferences to routine processing and staining.[6]

As theorised based on the study made by Patil et al., fructose present in natural fixatives creates a low pH environment which would result in a breakdown process to form aldehydes which then cross-link with tissue amino acids present for tissue fixation to occur. This possible fixation mechanism is similar to the action of formaldehyde, providing evidence that natural fixatives can be used as an alternative to formalin.[7,12]

A study conducted by Chittemsetti et al. compared the fixative ability of both khandsari and jaggery, sugar cane derivatives. Their results showed that tissue sections fixed in khandsari provided promising results regarding cytoplasmic staining, nuclear details and staining quality. This demonstrates that natural fixatives can replace the use of formalin.[13] There are several studies regarding alternative fixatives, and some of them showed promising results.

This article was accepted: 03 November 2022
Corresponding Author: Phyu Synn Oo
Email: phyusynnoo@imu.edu.my

However, formaldehyde is still used in daily clinical practice because most studies' results are obscure and conflicting and need robust research.

In modern health care, systematic reviews are used to appraise evidence, and information policy, construct guidelines and assess the cost-effectiveness of interventions.[14] This systematic review aims to elucidate the spectrum of natural fixatives and their efficacy in tissue preservation. With scientific evidence, this study can establish safer, eco-friendly natural fixatives alternatives to formaldehyde.

MATERIALS AND METHODS

Literature Search

The search strategy followed the Cochrane guidelines, Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), for systematic review.[14] The articles included in this review were extracted from PubMed, Ovid Medline and Google scholar databases, and backreferences of the articles till 31st March 2021. The internet search was also done to enhance the inclusion of the relevant articles. The studies which assessed and compared the efficacy of natural fixatives, such as honey, sugar, cane and khandsari, were included in this study. The titles and abstracts of articles were systematically screened by two independent researchers at all stages and identified eligible studies. The search included terms ('natural fixatives' OR 'sugar fixatives' OR 'honey fixatives' OR 'Jaggery fixative' OR 'sugar cane fixatives' OR 'khandsari fixatives') AND ('tissue morphology') AND ('nucleic acid' OR 'DNA' OR 'RNA') AND ('protein'). In addition, an internet search was also done using the above-mentioned keywords.

Study Selection

Studies were included if: (1) they were original studies that evaluated the use of natural fixatives in tissue samples (both human and animal) as an alternative to formaldehyde; (2) they were published in English. Studies were excluded if (1) they were review articles, case reports, case series, conference abstracts, editorials, letters to the editor and commentaries; (2) they were published in languages other than English.

Two independent authors (AYWL and PSO) screened titles and abstracts of all collected articles and then evaluated the full text of studies that met inclusion criteria. A third author (SNA) resolved disagreements at both screening stages. Eligible articles were included based on the pre-specified criteria, and references were screened to retrieve additional studies.

Quality Assessment of Included Articles

All studies were evaluated for quality assessment with NHLBI Study Quality Assessment Tools. The credibility of the knowledge produced, and the product's usefulness determined the quality of the evidence recorded.

Data Extraction

Extracted data included the name of the first author, publication year, country of the study, tissue type, fixative type, morphological analysis and protein and nucleic acid (DNA/RNA) level. One reviewer (AYWL) extracted the data and confirmed it by an independent reviewer (PSO).

RESULTS

Study Selection Process

Initially, 468 studies were recovered from three databases (PubMed: 295, Ovid Medline: 83, google scholar: 90). A total of 450 articles were excluded based on the exclusion criteria by screening the title and abstract. A total of 18 relevant articles were collected after excluding the duplication. Three articles were added from the google search. After reviewing full articles, six were excluded, as formaldehyde was not used as a control experiment, lacked histological analysis, deviated from standard tissue processing, or had incomplete result interpretation. Fifteen articles were finalised to review; data were collected and tabulated from each article, and results were verified and interpreted (Figure 1).

Study Characteristics

The present review included the studies published between 2008 and 2021. Based on geographical location, nine studies were from India, two from Oman and one from Nepal, Nigeria, Thailand and Turkey. All articles described the results of cross-sectional studies (Table I, Figure 2).

Nine studies (60%) tested the natural fixatives in human samples, while 6 (40%) studies used animal samples. Human tissue samples included oral mucosa, gingiva, lymphoid, salivary gland, fat, muscle, skin, endometrium, breast, placenta, uterus, omentum, suprarenal gland, stomach, lung, etc. Animal samples included the liver, kidney, brain, lung, heart, intestines, stomach, spleen, tongue, buccal mucosa, and brain. Sample sizes are from n = 5 to n = 90. One study did not mention the sample size. Eleven studies tested the fixation for up to 24 hours; two studies tested 24 to 72 hours, and two studies tested for a duration of stability of up to 6 months. Thirteen studies were conducted at room temperature except for milk, but two papers did not mention the temperature. Table I illustrates the studies' (n = 15) characteristics.

Natural Fixatives Used in Studies

Six out of 15 studies used more than one natural fixative in each study,[7,13,15-18] while nine used one fixative with different concentrations or diluted in different solutions.[6,8,12,19-24] The natural fixatives used in the studies were honey, jaggery, sugar and khandsari sugar, and their fixative efficacy excelled compared to formalin-fixed control specimens. Milk and ice were used as transport media as well. Twelve out of 15 studies included honey as a natural fixative alternative to formalin. The second most studied natural fixative was jaggery (n = 8) which is followed by sugar (n = 4) and other fixatives like milk or ice (n = 1) (Figure 3).

Different grades of commercial honey were used in the studies and were prepared with distilled water (DW), neutral buffer and ethanol. Dilutions from 1% to 100% were used in the studies.

Natural Fixatives in Tissue Fixation

Five out of 15 studies described gross fixation or changes during tissue microtoming (Table II). Chittemsetti et al. described the tissue folding and difficulty in preparing the sections,[13] but other studies revealed the brownish discolouration of fixed tissues by jaggery without interfering with histological findings.[6,7] For longer fixation time, Inyang

and Udonkang described that 20% and 50% of honey showed poor fixation after 72 hours, while 70%, 90% and 100% revealed good preservation for up to 6 months.[21]

Honey as an Alternative Fixative in Histopathology

Eleven out of 15 studies examined the histological features of tissues by Haematoxylin and Eosin (H&E) staining alone (Figure 3). One study examined the histological features of tissue by special stains[19,] and three studies used both H&E and special stains.[18,20,23]

Four studies used 10% honey to determine the histological analysis by H&E staining in rat tissues (liver, kidney and stomach) and human tissues (oral mucosa, endometrium, breast, placenta, uterus, omentum, suprarenal, stomach and lung).[8,12,20,23] The nuclear and cytoplasmic staining by H&E revealed a similar demonstration to that of formalin-fixed tissues. However, Lalwani et al. revealed more artefacts in processed and unprocessed honey-fixed tissues compared to formalin-fixed tissue samples. Out-of-focus areas and hyalinised tissues were reported in Lalwani's and Srii's studies[8,12] (Table II).

Six studies used 20% honey to demonstrate the histological feature by H&E staining. 20% honey-fixed tissues revealed good nuclear details and staining quality after 24 hours of fixation. However, after 6 months of fixation, cellular and nuclear clarity were decreased in 20% honey-fixed tissues compared to 10% formalin-fixed tissues. Cellular and nuclear shrinkage was also observed in H&E staining. The connective tissue staining by PAS and Mason Trichrome stains also revealed adequate but not optimal.18 Even though other researchers stated that a higher concentration of honey caused tissue shrinkage, a higher concentration of honey (50%-100%) was used in two studies. The nuclear and cytoplasmic staining revealed similar staining qualities of formalin-fixed tissues.[21,24] Sabarinath et al.[24] reported connective tissue homogenisation and background staining in honey-fixed tissues (Table II).

In the case of tissue fixation, Udongkang (2018) reported that 20% and 50% of honey revealed poor fixation after 72 hours, although nuclear and cellular staining revealed promising results. However, after 6 months, 70%, 90% and 100% honey demonstrated good tissue preservation (Table II).[21] The overall findings suggested that honey was a promising fixative for histological examination in routine histopathological practice.

Jaggery as an Alternative Fixative in Gross and Histological Examination

Eight out of 15 studies (53%) used jaggery as an alternative fixative and compared the tissue fixation efficiency and histological interpretation using H&E and special stains (Figure 3). One study used 20% jaggery diluted in DW,[16] and the remaining seven used 30% jaggery in human specimens and goat buccal mucosa tissues.[6,7,13,15,17,18,22] Patil (2013), Kuriachan (2017) and Sinha (2017) revealed that 30% of jaggery-fixed tissues demonstrated similar or superior results in H&E staining compared with that of formalin after 24 to 48 hours of fixation. 30% of jaggery-fixed tissues revealed good overall morphology with good nuclear and cellular outlines.[6,7,16] In shorter durations (1, 6 and 12 hours), 30% of jaggery-fixed tissues demonstrated superior to formalin-fixed tissues in histological examination.[17] However, other researchers revealed contrasting results on the fixation efficiency of jaggery in 20% or 30% in DW compared to formalin-fixed tissues. Chittemsetti et al.[13] revealed that 30% of jaggery-fixed tissues demonstrated significant differences in cellular outline, cytoplasmic and nuclear details, staining quality and overall morphology compared with that of formalin-fixed tissues. Imran et al.[15] also revealed that 30% of jaggery demonstrated cellular swelling and tissue autolysis after 24-hour fixation. Lam-ubol et al.[22] revealed that 30% of jaggery-fixed tissues demonstrated satisfactory stroma staining after 24-hour fixation, while there was mild nuclear condensation in inflammatory cells and separation of epithelial cells from each other and underlying connective tissues. 72-hour fixation with 30% jaggery revealed fibrous stroma hyalinisation. The staining integrity was reduced after a 6-month fixation with 30% jaggery with cellular and nuclear shrinkage. The connective tissue staining was not optimised with PAS and mason trichrome stain.[18] The fixative efficacy of jaggery was controversial in the studies (Table II).

Sugar as an Alternative Fixative in Histological Examination

The study's third most used alternative fixative (n = 4) was sugar. One study used 30% khandsari sugar, which is unrefined cane sugar,[13] while the other three studies used 20% sugar syrup in DW.[7,15,16] Human oral tissue specimens and goat buccal mucosa specimens were fixed with 20%–30% sugar solutions for 24 hours, and histological examination was done with H&E staining. Chitttemsetti et al.[13] revealed that 30% khandsari sugar-fixed tissues revealed promising staining quality except for cellular outline compared with formalin-fixed tissues. A similar result was reported with 20% sugar syrup as a fixative.[15] Patil et al. and Kuriachan et al. demonstrated controversial results with 20% sugar syrup. In those studies, sugar-fixed tissues revealed poor overall staining with a lack of cellular outline clarity and uneven staining.[7,16] Additionally, tissue folds and difficulty in preparing sections were reported in khandsari-fixed tissues[13] and in 20% sugar-fixed tissues7 (Table II).

Other Natural Fixatives

Milk and ice were also used as instant transport media for soft tissue biopsy, and the diagnostic value of tissues was assessed with H&E staining. Milk was kept at 5°C, and fixation was done at the same temperature. Fixation with milk and ice was done for 1, 6, 12 and 24 h, followed by 10% buffered formalin for 24 h. Milk-fixed tissues revealed poor tissue structures after 1 h fixation, while ice-fixed tissued revealed deterioration and loss of morphological details after 12 h fixation.[17]

Natural Fixatives with Special Stains in Histopathology

Four studies used special stains to demonstrate the extracellular matrix staining in natural fixatives fixed tissue. Al-Maaini and Bryant revealed that 1% honey demonstrated poorly stained collagen, reticulin, elastin and keratin, while 5%, 10% and 20% honey demonstrated a compatible staining quality with 10% formalin.[19] Alwahaibi et al.[20] also demonstrated similar results with 10% honey diluted for connective tissues; however, reticulin staining intensity with

Table I: Demographic characteristics of sexual assault victims

	Study ID	Natural Fixatives	Sample size	Type of sample	Parameter analysis	Duration	T°	pH	Gross Morphology	Outcomes compared to formaldehyde		
										Histology (H&E)	Histochemistry	Immunohistochemistry
1	Al-Maaini, 2008, Oman	Honey	Not mentioned	Animal	liver, kidney, brain, lung, heart, bowel, stomach, spleen and tongue	24 hr	RT	Not mentioned	Not mentioned	Not done	1% honey → satisfactory result in MT 5%, 10% and 20% honey → satisfactory in VVG, GS, MT and Miller's elastic stain	Not done
2	Alwahaibi, 2021, Oman	Sumer honey Date honey	N = 81	Animal	liver, kidney & stomach	24 hr	RT	10% Sumer honey 3.56 10% Date honey 5.15 10% Netural buffer 7.25, 7.27 10% Alcoholic honey 5.1, 5.8	Satisfactory	Satisfactory	Satisfactory result with JMS, PAS and GS	Satisfactory (Vimentin)
3	Chittemsetti, 2018, India	Jaggery Khandsari	N = 90	Human	Oral surgical specimens	24 hr	RT	Not mentioned	Difficulty in in microtone	Satisfactory Khandsari> Jaggery	Not done	Not done
4	Imran, 2020, India	Honey Sugar Jaggery syrup	N = 40	Human	Human Oral tissues	24 hr	RT	4.5 – 5.5	Not mentioned	Satisfactory Formalin> sugar> honey> jaggery	Not done	Not done
5	Kuriachan, 2017, India	Honey Jaggery Sugar	N = 40	Human	human gingival tissue	24 hr	RT	Not mentioned	Not mentioned	Satisfactory Honey> Jaggery> formalin> sugar	Not done	Not done
6	Lalwani, 2015, India	Unprocessed honey Processed honey	N = 36	Human	oral epithelium, lymphoid, salivary gland, fat, muscle and skin	24 hr	RT	10% unprocess honey 3.6 10% process honey 5.05	Not mentioned	Satisfactory Processed honey> unprocessed honey	Not done	Not done
7	Lam-ubol, 2018, Thailand	Jaggery	N = 40	Human	Oral tissues	24 hr & 72 hr	RT	Not mentioned	Not mentioned	Satisfactory	Not done	Not done
8	Özkan, 2011, Turkey	Pine honey	N = 21	Human	endometrium, breast, placenta, uterus, omentum, suprarenal, stomach and lung	24 hr	RT	4.8 – 5.0	Not mentioned	Satisfactory trichrome)	Satisfactory (Gomori's	Satistifactory (Ki-67, Vimentin)
9	Patil, 2013, India	Honey Sugar syrup Jaggery syrup	N = 5	Animal	buccal mucosa	24 hr	RT	Not mentioned	Brownish colour in jaggery solution	Satisfactory	Not done	Not done

cont..... pg 101

cont from..... pg 100

Table I: Demographic characteristics of sexual assault victims

	Study ID	Natural Fixatives	Sample size	Type of sample	Parameter analysis	Duration	T°	pH	Outcomes compared to formaldehyde			
									Gross Morphology	Histology (H&E)	Histochemistry	Immunohistochemistry
10	Patil & Rao, 2015, India	Honey Jaggery Milk Ice	n = 80	Animal	buccal mucosa	1, 6, 12, 12, 24 hr	RT	Not mentioned	Not mentioned	Satisfactory	Not done	Not done
11	Patil, 2015, India	Jaggery Honey	n = 42	Animal	Fresh goat meat (buccal mucosa)	48hr – 6 months	Not mentioned	Not mentioned	Not mentioned	Satisfactory	Satisfactory (PAS, MT)	Not done
12	Sabarinath, 2014, India	Honey	n = 30	Human	Human Oral çissues (pericornitis, percoronal abscess)	24 hr	Not mentioned	Not mentioned	Not mentioned	Satisfactory	Not done	Not done
13	Sinha, 2017, India	Jaggery solution	n = 65	Human	Human pathologica specimens	24 - 48 hr	RT	4.5 – 5.5	Not mentioned	Satisfactory	Not done	Not done
14	Srii, 2016, Nepal	Bee honey	n = 60	Human	gingiva and pericoronal region	24 hr	RT	4.5 - 5	Brown tint tint in jaggery solution	Satisfactory	Not done	Not done
15	Udongkang, 2018, Nigeria	Honey	n = 10	Animal	heart, intestine, lungs, kidneys, and brain	48 hr, 1 week, 2 weeks, 1 month, 3 months 6 month	RT (27'C)	4	20% and 50% honey → poor fixation after 72 hours 70%, 90% and 100% honey → good preservation after 6 months	Satisfactory	Not done	Not done

Footnote RT = Room temperature, MT = Masson trichrome stain, VVG = Van Gieson Stain, GS = Gordon & Sweet's reticulin stain, JMS = Jones Methenamine silver stain, PAS = Periodic acid–Schiff stain

Table II: Findings of natural fixatives-fixed tissues compared to formalin

	Study ID	Natural Fixatives	Type of tissues	Type of stain used	Findings		
					Tissue sectioning	Histology	Artefacts in alternative fixatives-fixed tissues
1	Al-Maaini, 2008, Oman	Honey	liver, kidney, brain, lung, bowel, stomach, spleen and tongue	1) VVG 2) Miller's elastin 3) GS 4) MT	Not mentioned	- Excellent connective tissue staining in honey-fixed tissues comparable to formalin-fixed control tissues	Not mention
2	Alwahaibi, 2021, Oman	Sumer honey Date honey	liver, kidney & stomach	1) H&E 2) JMS 3) GS 4) PAS 5) IHC	Not mentioned	- Satisfactory overall quality of staining in honey-fixed tissues - Inadequate cytoplasmic staining in neutral buffered honey and 10% honey compared - Weak reticulin fibres staining with Gordon and Sweets method in all honey groups	Absence of red blood cell staining
3	Chittemsetti, 2018, India	Jaggery Khandsari	Oral surgical specimens	1) H&E	Tissue folds and difficulty in preparing sections	- Formalin is superior to Khandsari sugar and jaggery in histological staining. - Khandsari sugar is superior to jaggery in histological staining.	Homogenization of tissues, loss of structure differentiation in jaggery
4	Imran, 2020, India	Honey Sugar Jaggery syrup	Human Oral tissues	1) H&E	Not mentioned	- Good staining quality in sugar and honey-fixed - Cellular swelling and tissue autolysis in jaggery-fixed tissues	Not mentioned
5	Kuriachan, 2017, India	Honey Jaggery Sugar	human gingival tissue	1) H&E	Brittleness of tissues fixed in sugar	- Superior tissue staining in honey and jagger-fixed tissues - Poor overall staining and a lack of clarity of cell outline in sugar-fixed tissues	Not mentioned
6	Lalwani, 2015, India	Unprocessed honey Processed honey	oral epithelium, lymphoid, salivary gland, fat, muscle and skin	1) H&E	Not mentioned	- Adequate tissue morphology and staining for diagnosis in honey-fixed tissues - Processed honey demonstrated better results than unprocessed honey.	Out-of-focus area and slit-like spaces in epithelial tissues and hyalinized collagen fibres
7	Lam-ubol, 2018, Thailand	Jaggery	Oral tissues	1) H&E	Not mentioned	- Satisfactory tissue morphology and staining quality in Jaggery-fixed and formalin-fixed tissues. - Mild chromatin condensation of inflammatory cells, hyalinization of fibrous stroma in jaggery-fixed tissues.	Epithelial separation from each other (acanthosis) and underlying connective tissue.
8	Özkan, 2011, Turkey	Pine honey	Endometrium, breast, placenta, uterus, omentum, suprarenal, stomach and lung	1) H&E 2) GT	Not mentioned	- Weak nuclear and cytoplasmic details in endometrial tissues - Well-preserved cell morphology, cytoplasm and nuclear antigen preservation and staining in other types of tissues	Not mention
9	Patil, 2013, India	Honey Sugar syrup Jaggery syrup	Buccal mucosa	1) H&E	Sugar syrup - difficulty in sectioning	- Satisfactory staining with H&E in all types of fixatives - Uneven staining in honey- and jaggery-fixed tissues	Sugar syrup - tissue folds

cont..... pg 103

cont from..... pg 102

Table II: Findings of natural fixatives-fixed tissues compared to formalin

	Study ID	Natural Fixatives	Type of tissues	Type of stain used	Findings		
					Tissue sectioning	Histology	Artefacts in alternative fixatives-fixed tissues
10	Patil & Rao, 2015, India	Honey Jaggery Milk Ice	buccal mucosa	1) H&E	Not mentioned	- High-quality preservation of tissue morphology and staining quality in 30% jaggery	Not mentioned
11	Patil, 2015, India	Jaggery Honey	Fresh goat meat (buccal mucosa)	1) H&E 2) PAS 3) MT	Honey and Jaggery fixed tissues - fragile and need attention in sectioning	- Cellular and nuclear shrinkage after six months of fixation in jaggery and honey - Satisfactory overall histomorphology with H&E stain in all fixatives - Adequate connective tissue staining in jaggery and honey-fixed tissues	Not mentioned
12	Sabarinath, 2014, India	Honey	Human Oral tissues (pericornitis, percoronal abscess)	1) H&E	Not mentioned	- Satisfactory morphology and staining with H&E in honey	Homogenization of collagen fibres in honey-fixed tissues
13	Sinha, 2017, India	Jaggery solution	Human pathological specimens	1) H&E	Difficulty during sectioning because of hard tissues in jaggery-fixed tissues.	- Satisfactory and comparable morphology and staining quality in jaggery and formalin-fixed tissues - Better nuclear details in jaggery-fixed tissues.	Not mentioned
14	Srii, 2016, Nepal	Bee honey	Gingiva and pericoronal region	1) H&E	Not mentioned	- No nuclear and cytoplasmic size change between honey- and formalin-fixed tissues	Hyalinization of collagen fibres in honey-fixed tissues
15	Udongkang, 2018, Nigeria	Honey	Heart, intestine, lungs, kidneys, and brain	1) H&E		- Good histological staining and cellular structures of tissues in higher concentrations of honey beyond 48 hours.	Not mentioned

Footnote: H&E= Haematoxylin and eosin, MT = Masson trichrome stain, VVG = Van Gieson collagen Stain, GS = Gordon & Sweet's reticulin stain, JMS = Jones Methenamine silver stain, PAS = Periodic acid–Schiff stain, IHC = Immunohisochemistry, GT = Gomori's trichrome muscle and collagen stain

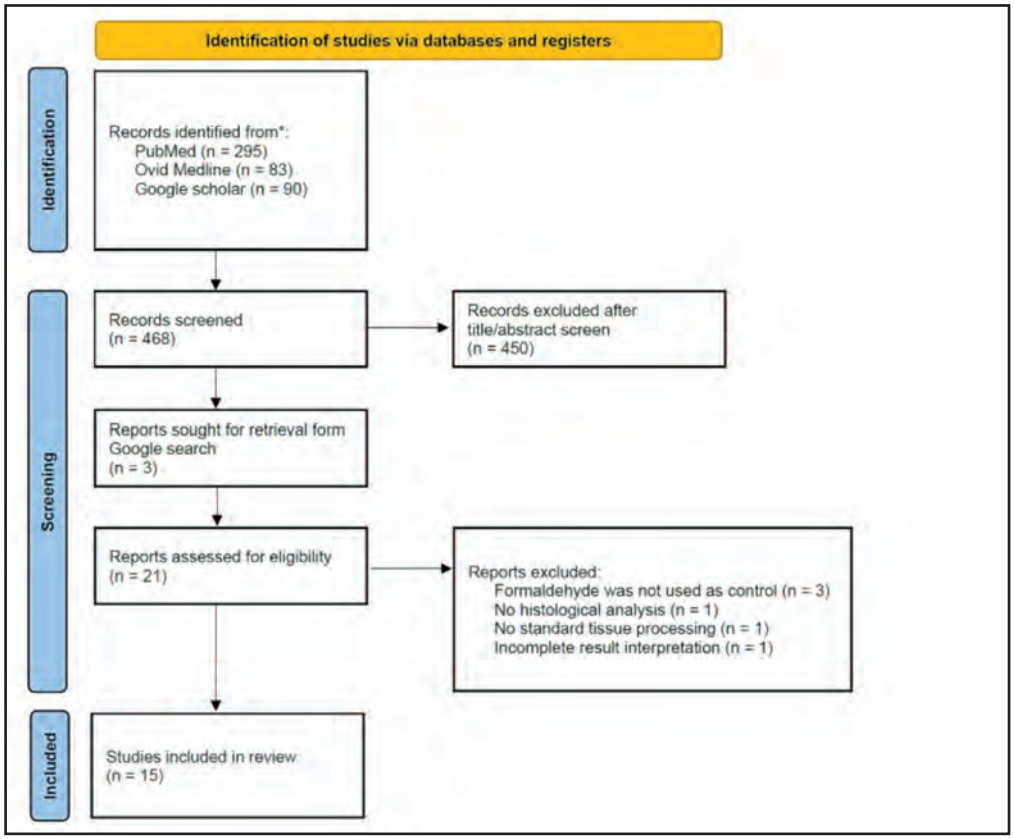

Fig. 1: PRISMA flow diagram for included studies

Gordon and Sweets method. However, Özkan et al. and Patil et al. revealed that 10% honey, 20% honey and 30% jaggery syrup demonstrated weak collagen and reticulin staining intensity compared to that of 10% formalin[18,23] (Table II).

Natural Fixatives in Immunohistochemistry

Two studies reported the vimentin and Ki-67 staining by immunohistochemistry in honey-fixed tissues showing similar findings to that of 10% neutral buffered formalin.[20,23]

Natural Fixatives and Artefacts

Seven studies addressed the artefacts in natural fixatives-fixed tissues compared to 10% formalin (Table II). Honey-fixed tissues failed to demonstrate red blood cell staining in liver and kidney samples.20 Out-of-focus area in epithelial tissues, slit-like spaces at the epithelium basement membrane[8] and homogenisation of collagen fibres[8,12,24] were found in honey-fixed tissues compared to 10% formalin-fixed tissues. In contrast with the findings of Patil et al., three studies addressed the artefacts in jaggery-fixed tissues; homogenisation of tissues, loss of tissue structure differentiation,[13] and epithelial separation from each other (acanthosis) and underlying connective tissues.[22] One study described tissue fold artefacts in sugar srup-fixed tissues[7] (Table II).

DISCUSSION

This is the first study to evaluate the natural fixatives as an alternative to formalin in a systematic review to the best of our knowledge. Fixation is a critical step in tissue processing for histopathological examination and archival preservation by preserving the cellular architecture and composition of cells in the tissues. Fixation also preserves proteins, carbohydrates and other bio-active components in their relationship to cells.[25] An ideal fixative should be able to harden the tissue components and prevent decomposition, bacterial putrefaction and autolysis. Routinely used 10% formalin contains 3.7% formaldehyde in water with 1% methanol. When the tissues are immersed in formalin, methanol initially causes dehydration, hardening the tissues and membrane, followed by a cross-linking phase with protein, mediated by aldehyde.[26] Formalin is the most widely used fixative in histopathological practice worldwide because of its convenience in handling, accuracy, adaptability and cost-effectiveness. However, as formalin is considered a toxic and carcinogenic substance to humans, many questions have been raised to seek an alternative fixative to replace formalin in histopathological practice.

Natural fixatives act by various mechanisms to best preserve the tissues. A higher level of fructose composition in honey, jaggery and sugar suggested the possible fixation mechanism by breaking down fructose into aldehyde and developing the cross-link with tissue amino acid.[7] Moreover, honey's antibacterial, acidic and dehydrating properties, whilst the cytoprotective and antioxidant activity of jaggery, supported the potential fixating properties of natural fixatives.[6]

Honey was the most frequently used alternative fixative to replace formalin because of its antibacterial effects. Bacteria

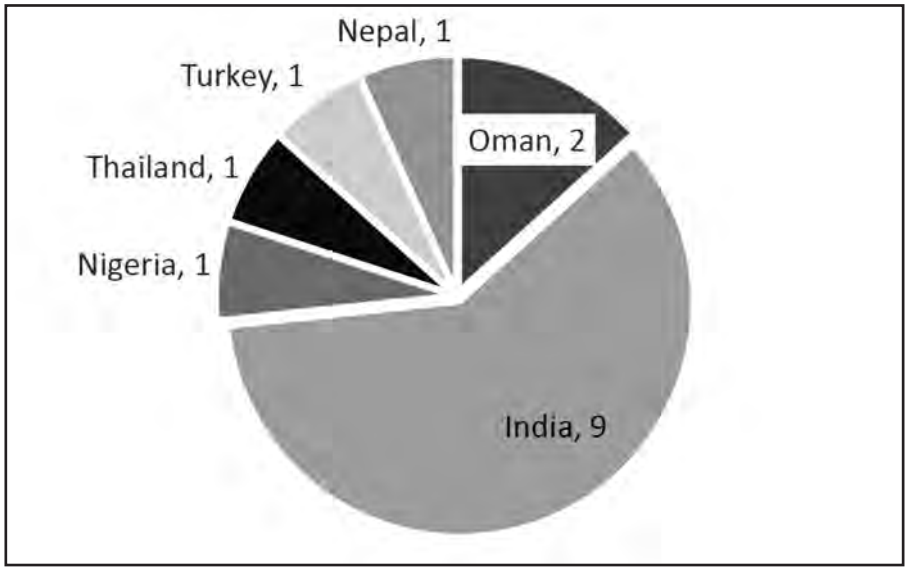

Fig. 2: Geographic distribution of studies included in the systematic review

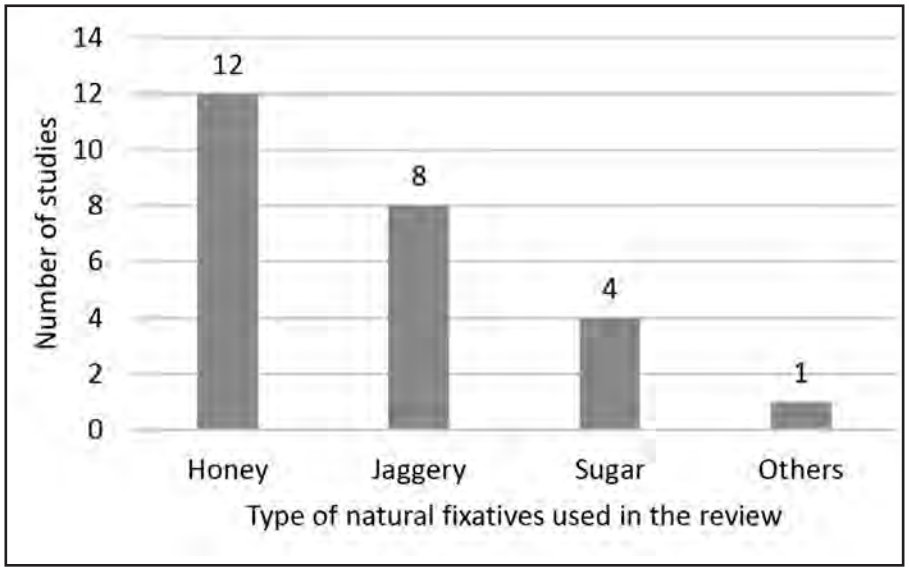

Fig. 3: Natural fixatives used in the systematic review

cannot survive in honey because of acidic pH and high sugar content, which dry out the bacteria with an osmotic effect.[27] Moreover, glucose peroxidase is released in diluted honey and oxidises glucose to produce hydrogen peroxide, inhibiting certain bacteria's growth.[27]

Eleven studies showed that honey provided the proper tissue fixation and compatible staining intensity with formalin in H&E stain, special stains and immunohistochemistry staining.[7,8,12,15-19,21,23,24] However, the concentration of honey was inconsistent in the studies. The argument may be due to the different chemical compositions of honey depending on the botanical and geographical origin.[27] Honey was diluted in distilled water, neutral buffer and alcohol from 1% to 90%. The studies agreed the lower concentration of honey gave better antiseptic actions because of the slow-release hydrogen peroxide at low concentrations. Honey also showed inhibitory effects on gram-positive and gram-negative bacteria, with a minimum concentration of 0.5% to a maximum concentration of 40%, depending on the type and production of honey.[28] The fermentation process was produced in diluted honey at room temperature. However, mould development was found on paraffin block and in diluted honey solution at room temperature.[19] Thus, more studies are required to optimise the honey concentration for tissue fixation based on kill kinetics against microorganisms in honey or keep the process in cold storage.

Fixation is a complex series of chemical events and differs for the different groups of chemical substances found in tissues.[29] The fixation speed depends on the rate of diffusion of the fixative into the tissue and the rate of chemical reaction with

various components. In practice, it is estimated that the fixation process requires at least 1 hour/mm of tissue thickness; however, the tissues are routinely fixed for 24–48 hours.[1] All included studies used the small piece of tissue samples (5 mm) in their studies with a 1:10 ratio of tissue and fixatives for 24–48 hours. It provided that natural fixatives could penetrate the small tissues and complete the fixation process. However, larger tissue samples were not pointed out. Piątek-Koziej et al.[30] revealed that 10% honey in DW was unsuitable for fixing large tissue samples like whole swine hearts, but 10% honey in absolute alcohol gave satisfactory fixation with tissue shrinkage. These findings suggested that an alcohol-based honey solution would provide a more promising result, but optimal alcohol dilution needs to be figured out.

Jaggery and sugar are mainly composed of sucrose. It was hypothesised that jaggery and sugar might preserve the tissue by breaking down sucrose at low pH, producing aldehydes and cross-linking with tissue amino acids.[7] This hypothesis explains why sugar and jaggery are best to fix at low pH (3.6–5.8) while formalin is best to fix at neutral pH.[6,15] The studies revealed promising results in tissue fixation with 20% or 30% jaggery or sugar diluted in DW.[6,7,13,15-18,22] 30% of jaggery fixatives showed superior cellular and nuclear staining compared to formalin or other natural fixatives.[6,7,16-18,22] Still, their findings were not aligned with Chittemsetti et al. and Imran et al., who showed cellular swelling and tissue autolysis in jaggery-fixed tissues.[13,15] It may be due to the different techniques used in cane processing to remove colour and impurities, which affect the number of polyphenols in sugar and jaggery.[31]

With natural fixatives, the authors focussed mainly on tissue fixative and histological staining. Only two studies tested the protein expressions by immunohistochemistry,[20,23] but protein levels and nucleic acid levels were not tested. Although six studies were conducted with human tissue biopsies, only histomorphological examinations were demonstrated. Specific disease and diagnostic variation were not evaluated between formalin-fixed and alternative fixatives-fixed tissues.

All the natural substances, honey, sugar, jaggery and khandsari sugar, gave promising results in small tissue fixation. Pricewise, honey is more expensive than formalin, but jaggery is cheaper and costs 1/6th of honey. Because of economic reasons, jaggery is more favourable than honey even though its chemical composition on antibacterial effects is limitedly known.[6,7,13,15,16,22]

CONCLUSION

Natural substitutes like honey, jaggery and sugar are boons when the health hazards of formalin are considered. Those natural fixatives provided promising results in small tissue fixation and histological staining. More studies should be done to explore the optimal dilutant and dilution for locally available natural fixatives. There are no studies regarding nucleic acid and protein levels in natural fixatives-fixed tissues, and these areas need further exploration. The penetrating power of the natural fixatives in different tissues also needs to be examined, and larger tissue samples should be tested for fixation. No disease tissue or diagnostic interpretation was made for alternative fixatives-fixed tissues. Future studies should be done in disease tissues to determine whether alternative fixatives can overcome the formalin pigments and artefacts or not. The authors of this article are currently working on an ongoing project that evaluates the effect of natural fixatives in histochemical staining as an alternative to formalin. By using natural fixatives, we aim to derive a safe and pleasant working environment for healthcare professionals.

ACKNOWLEDGEMENT

This study was supported by Biomedical Science Program, International Medical University, Kuala Lumpur, Malaysia (No. BMSc I-2021 (16) to Phyu Synn Oo).

CONFLICT OF INTEREST

There is no actual or potential conflict of interest in relation to this article.

REFERENCES

1. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002; 161(6): 1961-71.
2. Musiał A, Gryglewski RW, Kielczewski S, Loukas M, Wajda J. Formalin use in anatomical and histological science in the 19th and 20th centuries. Folia Med Cracov 2016; 56(3): 31-40.
3. Shetty JK, Babu HF, Hosapatna Laxminarayana KP. Histomorphological assessment of formalin versus nonformalin fixatives in diagnostic surgical pathology. J Lab Phys 2020; 12(4): 271-5.
4. Cogliano VJ, Grosse Y, Baan RA, Straif K, Secretan MB, Ghissassi FE, et al. Meeting report: summary of IARC Monographs on formaldehyde, 2-butoxyethanol, and 1- tert -butoxy-2-propanol. Environ Health Perspect 2005; 113(9): 1205-8.
5. Mundt KA, Gentry PR, Dell LD, Rodricks JV, Boffetta P. Six years after the NRC review of EPA's draft IRIS toxicological review of formaldehyde : regulatory implications of new science in evaluating formaldehyde leukemogenicity. Regulat Toxicol Pharmacol 2018; 92: 472-90.
6. Sinha N, Nayak M, Sunitha J, Dawar G, Rallan N, Gupta S. Comparitive efficacies of a natural fixative with a conventional fixative. J Oral Maxillofac Pathol 2017; 21(3): 458.
7. Patil S, Premalatha B, Rao RS, Ganavi B. Revelation in the field of tissue preservation - a preliminary study on natural formalin substitutes. J Int Oral Health 2013; 5(1): 31-8.
8. Lalwani V, Surekha R, Vanishree M, Koneru A, Hunasgi S, Ravikumar S. Honey as an alternative fixative for oral tissue: an evaluation of processed and unprocessed honey. J Oral Maxillofac Pathol 2015; 19(3): 342.
9. Muddana K, Muppala JNK, Dorankula SPR, Maloth AK, Kulkarni PG, Thadudari D. Honey and olive oil as bio-friendly substitutes for formalin and xylene in routine histopathology. Indian J Dental Res 2017; 28(3): 286.
10. Wijesinghe M, Weatherall M, Perrin K, Beasley R. Honey in the treatment of burns: a systematic review and meta-analysis of its efficacy. N Z Med J 2009; 122(1295): 47-60.
11. Zainol MI, Mohd Yusoff K, Mohd Yusof MY. Antibacterial activity of selected Malaysian honey. BMC Complement Altern Med 2013; 13(1): 129.
12. Srii R, Marla V. BEE honey as a locum for routine formalin fixative. Int. J. Sci. Res 2016; 5(10): 2277-8179.

13. Chittemsetti S, Nallamala S, Sravya T, Guttikonda VR, Manchikatla PK, Kondamari S. Natural substitutes for formalin: A boon to histopathology!! J Oral Maxillofac Pathol 2018; 22(1): 143.
14. Moher D, Liberati A, Tetzlaff J, Altman DG, for the PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ 2009; 339(jul21 1): b2535-b2535.
15. Imran M, Kumar D.R. S, Ahmed SA, Tanveer S. Eco-friendly natural fixatives - a substitute for formalin? Health Sci 2020; 12(06): 11906-9.
16. Kuriachan D, Suresh R, Janardhanan M, Savithri V, Aravind T, Thampy LM. Analysis of fixative properties of three eco-friendly substances: a comparison with formalin. Oral Maxillofac Pathol J 2017; 8(2): 79-84.
17. Patil S, Rao RS, Agarwal A, Raj AT. Instant transport media for biopsied soft tissue specimens: a comparative study. Scientifica 2015; 2015: 1-5.
18. Patil S, Rao R, Ganavi B, Majumdar B. Natural sweeteners as fixatives in histopathology: a longitudinal study. J Nat Sc Biol Med 2015; 6(1): 67.
19. Al-Maaini R, Bryant P. Honey as an alternative to formalin in the demonstration of connective tissue components. J Histotechnol 2008; 31(2): 67-72.
20. Alwahaibi N, Al Dhahli BS, Al Issaei H, Al Wahaibi L, Al Sinawi S. Efficiency of neutral honey as a tissue fixative in histopathology. bioRxiv [Preprint]. 2021.04.27.437988.
21. Inyang I, Udonkang M. Honey as fixative and safer substitute for formalin in histology. Int J Med Lab Res 2018; 3(3): 11-7.
22. Lam-ubol A, Kitrueangphatchara K, Putthanuparp T, Arayakhun R, Kwanthong R, Choonhawarakorn K. Nonformalin fixative agents: a comparative study of fixative efficacy and histomorphology. Int J Surg Pathol 2018; 26(8): 701-6.
23. Özkan N, Şalva E, Çakalağaoğlu F, Tüzüner B. Honey as a substitute for formalin? Biotechnic Histochem 2012; 87(2): 148-53.
24. Sabarinath B, Sivapathasundharam B, Sathyakumar M. Fixative properties of honey in comparison with formalin. J Histotechnol 2014; 37(1): 21-5.
25. Thavarajah R, Mudimbaimannar V, Rao U, Ranganathan K, Elizabeth J. Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol 2012; 16(3): 400.
26. Kiernan JA. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do. Micros Today. 2000; 8(1): 8-13.
27. White, JW, Jr., Landis W. Doner. Physical characteristics of honey. In: Crane, E, editor. Honey: a comprehensive survey. London: Heinemann; 1980. p. 157-206.
28. Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. Asian Pacif J Trop Biomed 2011; 1(2): 154-60.
29. Suvarna SK, Layton C, Bancroft JD. Bancroft's theory and practice of histological techniques. In: Bacnroft's theory and practice of histological techniques. 7th ed. Oxford: Elsevier; 2019. p. 105-24.
30. Piątek-Koziej K, Hołda J, Koziej M, Tyrak K, Jasińska KA, Bonczar A, et al. Fixative properties of honey solutions as a formaldehyde substitute in cardiac tissue preservation. Folia Med Cracov 2019; 59(1): 101-14.
31. Harish Nayaka MA, Sathisha UV, Manohar MP, Chandrashekar KB, Dharmesh SM. Cytoprotective and antioxidant activity studies of jaggery sugar. Food Chem 2009; 115(1): 113-8.