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AQUEOUS BEETROOT DYE AS AN ALTERNATIVE TO HAEMATOXYLIN AND EOSIN IN THE DIAGNOSIS OF BREAST TUMOURS

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ABSTRACT

Breast tumours are heterogeneous diseases that result from changes in the cells and connective tissues of the breast. Different histological and histochemical stains have been employed to aid in the detection of these changes in routine diagnosis. The aim of this study was to assess the usefulness of aqueous beetroot dye in the histochemical staining of breast tumours. Thirty breast tumour blocks from the Histopathology Laboratory of the University of Calabar Teaching Hospital, Calabar were retrieved, sectioned and stained with Cole's haematoxylin and eosin and aqueous beetroot. The histological staining and 3-D image characteristics were analysed. The histological types of breast tumours were 7(23.3%) benign, of which 5(16.7%) were grade II benign lesion, 1(3.3%) was fibrocystic disease and 1(3.3%) was fibroadenoma. Among the 23(76.7%) malignant tumours, 15(56.7%) were grade 3, 3(10%) were B4 carcinomas and 3(10%) were B5 types. The sections stained with the red beetroot dye gave distinct metachromatic staining of the connective tissues of the cell membranes of the epithelial cells and other connective tissue cells, cytoskeleton, cytoplasm, basement membrane, and collagen fibres of the stroma in shades of green, purple, and black. The histological staining and 3-D image characteristics of the beetroot-stained benign and malignant tumours were similar to the haematoxylin and eosin-stained counterparts in terms of nuclear, cytoplasmic and connective tissue staining, 3-D surface plot features, and histological details (χ^2=1.200; p=0.549). This finding has shown that betalain pigments in beetroot dye gave good metachromatic staining of epithelial and connective tissue changes in breast tumours. Beetroot dye and 3-D surface plots when combined can serve as a cost effective, health and environment-friendly alternative staining method to routine haematoxylin and eosin in diagnosis of breast tumours in low resource areas.

KEYWORDS: Breast tumour, Beetroot dye, haematoxylin and eosin, 3-D image

INTRODUCTION

Breast cancers have caused millions of death among females with increasing incidences in developing and developed countries, including Nigeria (Ebughe et al., 2016; Jedy-Agba et al., 2016; Lukong, 2017). Breast tumour is a heterogeneous disease affecting the cells and extracellular matrix of the breast and differs greatly among affected persons (Yan, 2013). Most breast tumours are attributed to gene mutations associated with the pathologic alterations of these epithelial cells and connective tissues (Young et al., 2006; WHO, 2016).

In Calabar, about 36 in every 100,000 women were reported to be diagnosed with breast cancer (Ebughe et al., 2016). All diagnoses were carried out with H&E method and most patients cannot afford the cost of the test due to poor resources. Also, H&E method utilizes chemicals that are hazardous to human health and the environment (Malhotra et al., 2010; Al-mura, et al., 2012). Most importantly, H&E method does not demonstrate detailed connective tissue or extracellular matrix changes (Wu et al., 2018). Hence, the need for a method that can highlight these extracellular matrix changes in breast tumours. As an alternative, this study utilized the use of aqueous beetroot dye as a

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histochemical stain to demonstrate the epithelial and connective tissue changes in benign and malignant breast tumours.

Beetroot (Beta vulgaris) is the taproot of the beet plant (Cheng et al., 2014; Singnarpi et al., 2017; Kumar et al., 2015). Beetroot is red in colour and has wide applications as food, food colourant, medicine, and stain for parasites, ova, buccal smears, and tissues of rats (Cheng et al., 2014; Singnarpi et al., 2017; Kumar et al., 2015; Tasneem & Hage, 2016; Udonkang et al., 2018). A study by Udonkang et al. (2018) showed that beetroot dye can stain the cytoplasm, smooth muscles, keratin, mucins, and collagen fibres in tissues of albino rats with shades of colours ranging from brown, pink, and green to black.

A three-Dimensional (3-D) surface plot was used because of its usefulness in revealing detailed changes in the cells and tissues at the molecular level. The interaction of the molecules in the cells and tissues with the stains leads to generation of light signals which are projected as 3-D images that are comparable with the histopathological features (Wu et al. 2018). This will aid in revealing the cell and extracellular matrix heterogeneity of breast tumours (Turashvili and Brogi, 2017).

Hence, the combination of beetroot staining with digital 3-D surface plots projections was to establish the use of locally available and cost effective dye in providing quality and accessible diagnostic healthcare in low resource areas.

MATERIALS AND METHODS

Study area

This study was carried out in the University of Calabar Teaching Hospital, a hospital-based cancer registry in Calabar, Cross River State. The hospital is located within Calabar Metropolis, made up of Calabar Municipal and Calabar South Local Government areas. Apart from receiving cases from the Metropolis, the hospital is a health referral center to other Local Government Areas of Akpabuyo, Bakassi, Odukpani and Biase.

Selection of tissue blocks/data collection

The study was carried out using thirty female breast tissue blocks from the Histopathology Laboratory, University of Calabar Teaching Hospital, Calabar. The blocks consisted of 1 normal tissue, 7 benign and 22 cancer-positive tissues. Data on type and histological grade of tumours were obtained. Ethical approval was obtained from the Research Ethics Committee of the University of Calabar Teaching Hospital, Calabar with Number UCTH/HREC/33/627.

Histological tissue preparation/staining

The selected breast tissue blocks were sectioned with a rotary microtome at 4 micrometers. The sections were floated out unto glass slides, dewaxed in xylene, and hydrated through descending grades (absolute, 95%, 70%) of ethanol and distilled water before staining in Cole's haematoxylin and eosin staining method.

Plant material, preparation and staining

Beetroots were obtained from Marian market, Calabar. The beetroots were authenticated at the Botany Department, University of Calabar, Calabar. The beetroots were washed with water and the skin peeled with a knife. The Beetroot solution was prepared by weighing 300g of the beetroot with a digital weighing balance, blended and dissolved in 100ml of distilled water. After preparation, dewaxed and hydrated tissue sections were stained with aqueous beetroot staining solution for 1 hour 30 minutes. All slides were rinsed in tap water, dehydrated, cleared, air-dried and viewed with an OMAX 40X-2500X light microscope (China) and photographs were taken with AmScope (MD500) digital camera (USA). Surface plots of 3-D images were produced similar to study by Wu et al. (2018).

Statistical/Image analysis

The results on types of tumours were presented as percentages. The comparison of the staining characteristics was calculated with Chisquare test using Statistical Package for Social Sciences (SPSS) version 20 (Armonk, New York: IBM Corporation). AmScope (MD500) digital camera software (USA) was used for image analysis to obtain surface plots. The surface plots were produced using the surface plot tool under the Process Menu of AmScope software manual. The surface plots of each photomicrograph produced were analysed. The level of probability was set at greater than or equal to 0.05 for results to be statistically significant.

RESULTS

The thirty tissues used were 1(3.3%) normal tissue, 7(23.3%) benign (comprising 5(16.7%) grade II benign lesions, 1(3.3%) fibrocystic disease and 1(3.3%) fibroadenoma) and 22(73.3%) malignant tumours (comprising 15(50%) grade 3 invasive ductal carcinomas, 4(13.3%) grade 2 carcinomas and 3(10%) B5 carcinomas) (Table 1).

Table 1: Histological types of breast tumours

Histological types	Benign	n(%)	Malignant	n(%)	Normal n(%)
1	Fibroadenoma	1(3.3)	Grade 2	4(13.3)	1(3.3)
2	Fibrocystic disease	1(3.3)	Grade 3	15(50)	
3	Grade 2 benign	5(16.7)	B5 malignant	3(10)	
Total 30(100)	**Total**	**7(23.3)**	**Total**	**22(73.3)**	**1(3.3)**

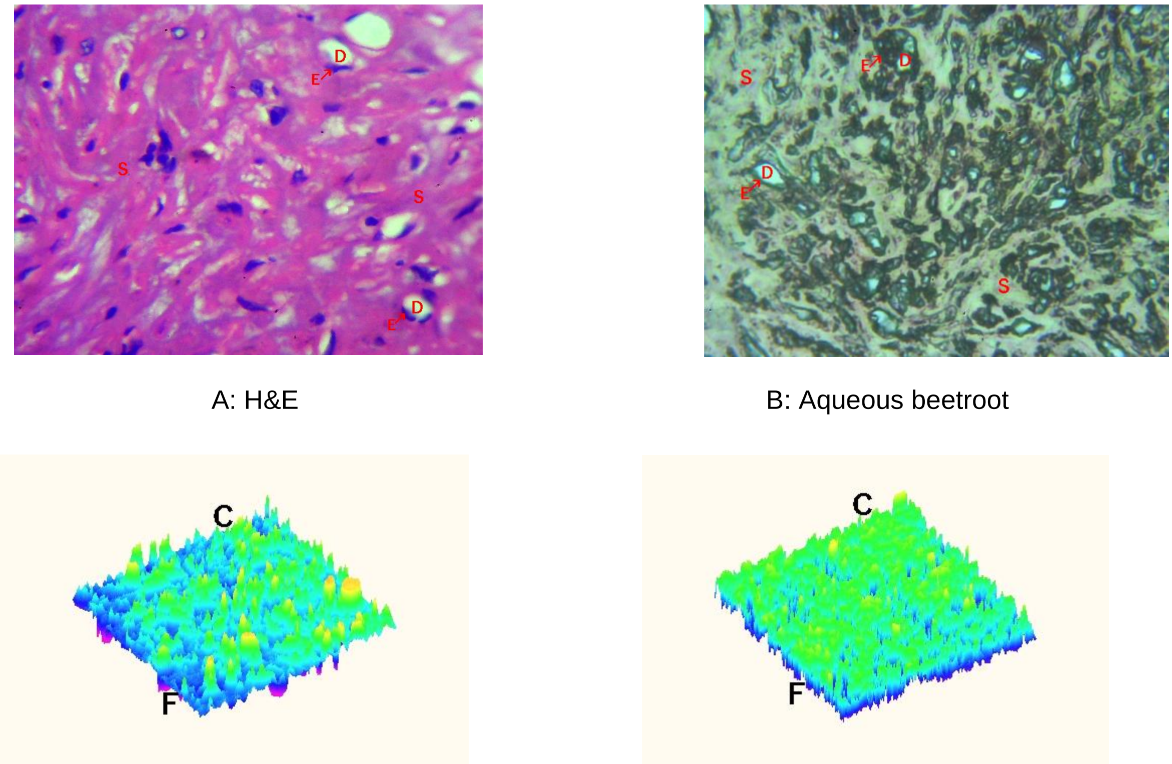
The photomicrograph of normal breast tissue is shown in Plate 1. The H&E-stained section showed normal ducts lined by epithelial cells embedded in smooth connective tissue stroma (Plate 1A). Plate 1B stained with aqueous beetroot dye showed darkly-stained connective tissues of epithelial cells lining the ducts and the lightly-stained smooth stroma. The 3-D surface plot of the H&E-stained section showed epithelial cells giving green to yellow signals at the surface and the stroma connective tissues giving blue to pink signals at the bottom (Plate 1C). Plate 1D is the 3-D surface plot of the aqueous beetroot dye with epithelial cells giving green to yellow signals at the surface and the stroma connective tissues giving blue signals at the bottom.

The fibroadenoma of the breast is shown in Plate 2. The section stained with H&E showed epithelial cells lining the duct with intact basement membrane and embedded in wavy connective tissue fibres in the stroma (Plate 2A). Plate 2B is fibroadenoma stained with aqueous beetroot showing darkly-stained connective tissue of epithelial cells surrounded by light green-stained intact basement membrane. The stroma is smooth and purple to light green-stained while the connective tissues of the cells in the stroma are darkly-stained. The surface plots of the H&E-stained and aqueous beetroot dye-stained sections show epithelial cells and other cells in the stroma giving similar strong distinct green-yellow-orange signals at the surface, while the connective tissues in the stroma emitted predominantly blue to pink signals at the bottom respectively (Plate 2C and 2D).

Plate 3 is fibrocystic disease of the breast. Plate 3A stained with H&E revealed thick connective tissue fibres in the stroma surrounding a large cystic duct. Plate 3B stained with aqueous beetroot dye showed predominant darkly-stained thick connective tissue fibres in the cystic duct and stroma. The surface plot of the H&E-stained and aqueous beetroot-stained sections show similar strong green-yellow-orange signals at the surface from epithelial cells and other cells in the stroma but the connective tissues in the stroma emitted intense blue-pink-red signals at the bottom respectively (Plate 3C and 3D).

Plate 4 is adenocarcinoma of the breast section. Plate 4A showed H&E-stained adenocarcinoma with desmoplastic epithelial cells in the duct surrounded by thick connective tissue fibres in the stroma. Plate 4B is stained with aqueous beetroot. The connective tissues of the cell membrane of the epithelial cells in the ducts and stroma had distinct dark staining with light green cytoplasm. The stroma had purple to light green prominent thick disoriented connective tissue fibres. The surface signals from epithelial cells and other cells in the stroma of the H&E-stained and aqueous beetroot-stained sections were green-yellow while the connective tissues in the stroma emitted moderate blue-pink signals at the bottom respectively (Plate 4C and 4D).

The histological staining and 3-D image characteristics of the beetroot-stained benign and malignant tumours were similar to the haematoxylin and eosin-stained counterparts in terms of nuclear, cytoplasmic and connective tissue staining, 3-D surface plot features, and histological details (χ^2=1.200; p=0.549, Table 2).



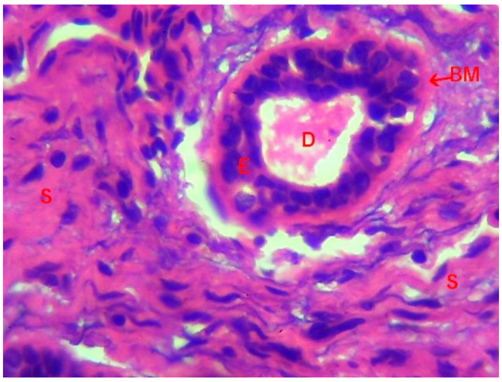
A: H&E

B: Aqueous beetroot

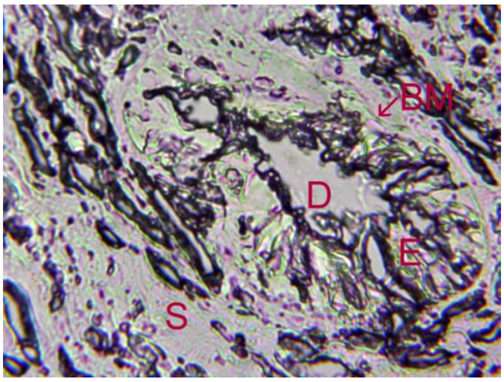
C: H&E

D: Aqueous beetroot

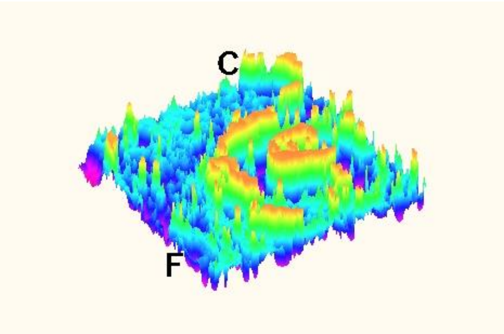
Plate 1: Photomicrographs and 3-D plots of normal breast tissue stained with H&E and aqueous beetroot. In 1A and 1B, the normal epithelial cells (E) in the ducts (D) are surrounded by normal connective tissue stroma (S). In 1C and 1D, the epithelial and other cells (C) gave green-yellow-orange signals at the surface while the collagen fibres (F) gave blue-pink signals at the bottom. (Magnificationx400).



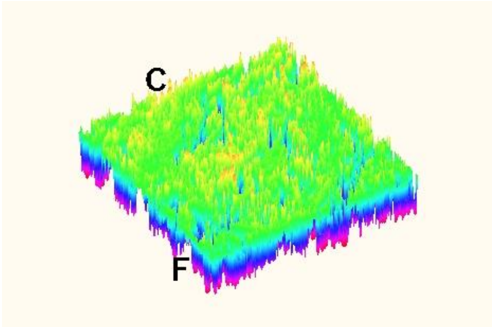
A: H&E



B: Aqueous Beetroot

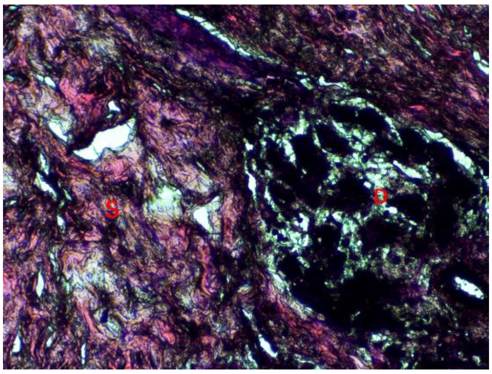


C: H&E

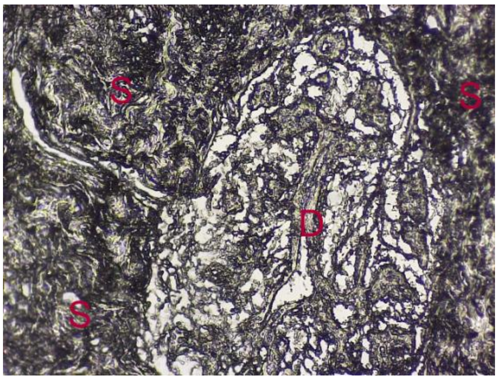


D: Aqueous beetroot

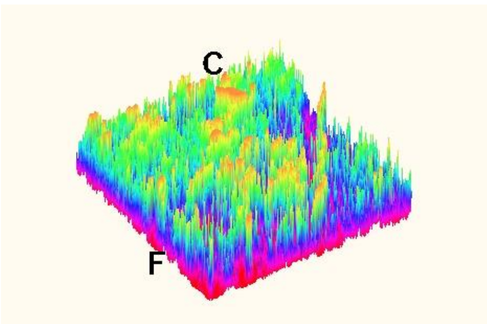
Plate 2: Photomicrographs and 3-D plots of benign fibroadenoma stained with H&E and aqueous beetroot. In 2A and 2B are epithelial cells (E) lining the ducts (D) surrounded by intact basement membrane (BM) and embedded in stroma (S). In 2C and 2D, the epithelial and other cells (C) gave green-yellow-orange signals at the surface while the collagen fibres (F) gave blue-pink signals at the bottom. (Magnification x400).



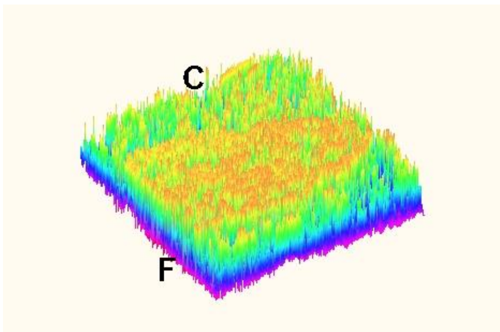
A: H&E



B: Aqueous beetroot

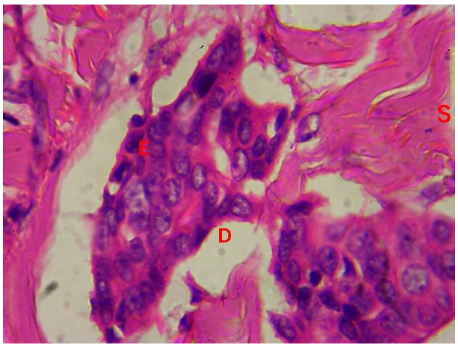


C: H&E

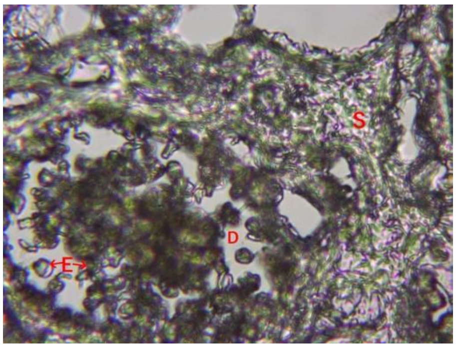


D: Aqueous beetroot

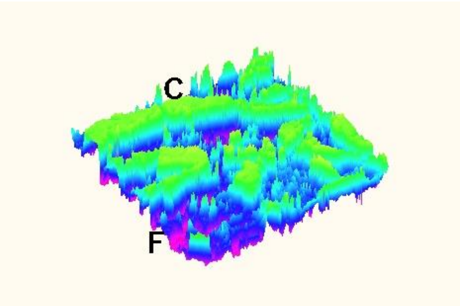
Plate 3: Photomicrographs and 3-D plots of fibrocystic disease stained with H&E and aqueous beetroot. Plates 3A and 3B show large cystic duct (D) surrounded by thick connective tissue stroma (S). In 3C and 3D, the epithelial and other cells (C) gave green-yellow-orange signals at the surface while the collagen fibres (F) gave blue-red-pink signals at the bottom. (Magnification x400).



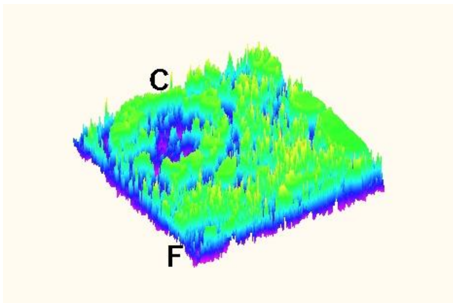
A: H&E



B: Aqueous beetroot



C: H&E



D: Aqueous beetroot

Plate 4: Photomicrographs and 3-D plots of adenocarcinoma stained with H&E and aqueous beetroot. The desmoplastic epithelial cells (E) in the ducts (D) are surrounded by thick connective tissue fibres in the stroma (S) in Plates 4A and 4B. In 4C and 4D, the epithelial and other cells (C) gave mostly green-yellow signals at the surface while the collagen fibres (F) gave blue-pink signals at the bottom. (Magnification x400)

Table 2: Comparison of histological staining and surface plot characteristics of normal breast tissue and breast tumours stain with H&E and beetroot

Staining parameters n=30	Nuclear	Cytoplasmic	Connective tissue fibres	Clarity	3-D plot Features	Total scores	Statistics
H&E	3	3	3	3	3	15	χ^2=1.200
Beetroot stain	2	1	3	3	3	12	p=0.549

Keys: Good=3, moderate=2 and poor staining=1

DISCUSSION

Breast tumours undergo diverse changes in their cells and connective tissues (Turashvili and Brogi, 2017). The routine H&E method showed morphological changes distinct to each tumour type. The cells and connective tissue changes of the ducts and stroma were prominent in the benign and malignant tumours. Several authors have reported that benign and malignant tumours undergo significant changes in the connective tissues especially in the collagen fibres morphology (Elasbali, 2019; Acerbi et al., 2015; Runa et al., 2017). These changes involve mostly increase deposition, thickening and reorganization of collagen fibres within the matrix. This increased collagen fibres production leads to tight cross-link formation that causes matrix stiffness. In turn, stiff matrix increases the risk of disease progression and aggression especially in malignancy.

The histochemical staining method adopted for this study was aqueous beetroot stain and its staining characteristics were compared with the routine H&E stain. As seen from the photomicrographs, beetroot gave distinct staining of connective tissues of the stroma and epithelial cells. There was detailed staining of the collagen fibres, basement membrane, cytoskeleton and cell membrane of the epithelial cells of the breast tissues.

The staining potential of beetroot is derived from the red colour of betalain pigments which is made up of betaxanthins and betacyanins (Udonkang et al., 2018). The red colour is derived from its dominant red-purple betacyanins because of their ability to have extended delocalization of electrons from copigmentation with betaxanthins pigments (Dumbrava et al., 2012; Galaffu et al., 2015; Solovchenko et al., 2019).

These betalain pigments also possess good staining properties due to their chemical composition. They are nitrogen-based pigments with cyclo-DOPA or amino or imino reactive groups (Dumbrava et al., 2012; Galaffu et al., 2015). These pigments have betalamic acid as their chromophore with a functional carboxyl (-COOH) group which provides a slightly acidic pH (Dumbrava et al., 2012). The acidic pH in turn confers specificity to basic tissue structures such as collagen fibres, basement membrane, cell membrane and cytoskeleton of the epithelial cells of the breast tissues (Udonkang et al., 2018).

The beetroot dye also gave metachromatic staining to the tissues by imparting varying colours of black, purple and green different from the red solution. This phenomenon of metachromasia may first be attributed to the bathochromic shift in the absorption spectra of the betalain pigments (D'mello, et al., 2016). This bathochromic shift was from a longer wavelength of red (525-740nm) to shorter wavelengths of green (520-565nm) and purple (380-430nm) and to achromatic black. Similar finding of bathochromatic shifts in betalain pigments from 580nm to 550nm was reported by Solovchenko et al. (2019). The phenomenon of bathochromic shift has also been shown in weakly acidic solutions of dyes that undergo intramolecular copigmentation (Solovchenko et al., 2019; D'mello, et al., 2016; Rodriguez-Amaya et al., 2019; Esquivel, 2016; Kammerer, 2016).

Another principle attributed to metachromasia is polymerization of polyamino-proteins of the dyes. The betaxanthins are rich in glutamine while the betacyanin and betanidin are rich in phenylalanine. Polymerization of these polyamino-proteins of betalains with tissue proteins has been found to contribute to colour change (Galaffu et al., 2015).

The cells and connective tissues of the breast in the normal tissue, benign, and malignant tumours gave similar 3-D signals in the H&E- and beetroot-stained sections. The epithelial cells and collagen fibres of the stroma in the normal tissues gave signals that revealed uniformity in their arrangement and morphology. The fibroadenoma tissues gave signals that demonstrated distortion in the arrangement of the epithelial cells while the red signals of collagen fibres in the stroma revealed increase in fibre density. The signals from the fibrocystic disease showed the disoriented arrangement of the epithelial cells as well as the marked change in the collagen fibres density and number. Lastly, the signals from the adenocarcinoma showed changes in the arrangement of the epithelial cells and increased disorientation in collagen fibre deposition. These findings are similar to works by Wu et al. (2018) and Wen et al. (2016) which show that tumours undergo complex changes in their extracellular matrix and 3-D images give details of these extracellular matrix and collagen structural changes. These changes are seen as light signals of different colours that are comparable with the histopathological features seen with H&E stains.

CONCLUSION

From the study, it has been shown that the cells and extracellular matrix notably the collagen fibres of the breast undergo distinct morphological changes in fibroadenoma, fibrocystic disease, and adenocarcinoma. Beetroot gave metachromatic staining of the breast tissues. It revealed in details the microstructural changes in the cells and collagen fibres. The 3-D surface plots of the beetroot-stained tissues were similar to the H&E-stained counterpart. The staining of the benign and malignant breast tissues showed that beetroot stain is valuable in the diagnosis of breast tumours. Thus, beetroot dye and 3-D surface plots when combined can serve as a cost effective, health and environment-friendly alternative staining method to routine haematoxylin and eosin in diagnosis of breast tumours in low resource areas.

Author contributions statement

MU and CE designed the study, did data interpretation, drafted and arranged the manuscript. AA, AE and II managed literature search and performed data collection and analysis. All authors read and approved the final version of the manuscript. MU is the guarantor of this work.

Disclosure statement

The authors declare that there is no conflict of interest.

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