Preparation and Characterization of *Beta Vulgaris* Peels and Pomace Dye Extracts as a Potential Natural Dye of Cotton Fabric

ROTICH KIPKOECH VINCENT

BSc

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Analytical Chemistry of Moi University

December 2021

DECLARATION

Declaration by the student

I, Rotich K. Vincent declare that this thesis is my original work and not a duplication of similar published work of any scholar for academic purpose as partial requirement of any university or institution. The citations, quotations and summaries in this thesis that are not mine have been duly acknowledged.

Signature…… Date 15th September, 2021…….

Rotich Kipkoech Vincent;

(MSC/ACH/04/18)

Declaration by the supervisors

This thesis has been submitted for examination with my approval as Moi university supervisor.

Signature…… Date……29/9/21……

Dr. Phanice Wangila

Department of Physical Sciences, University of Kabianga

Signature……. Date……29/09/2021……

Dr. Jackson Cherutoi

Department of Chemistry & Biochemistry, Moi University

ACKNOWLEDGEMENT

Foremost, I thank the Almighty God for His protection, love, wisdom, guidance, endurance and strength in research and writing this thesis. Moreover, I must express my heartfelt gratitude to my supervisors and lecturers Dr. Wangila Phanice and Dr. Cherutoi Jackson for their immense wisdom through fruitful guidance and advice right from course work, research and writing this thesis. May the Lord bless you abundantly. Iam extremely thankful to the Africa Center of Excellence in Phytochemical, Textile and Renewable Energy (ACEII PTRE) leadership led by Prof. Ambrose Kiprop and the secretariat for the financial support and greatly enabling me undertake this course and the research project. Special thanks to World Bank for funding us through ACE II-PTRE program. I cherish the lab technicians of Chemistry & Biochemistry, and Biological department laboratories, Moi University, School of Science and Aerospace studies for their unwavering guidance at budgeting, keeping of our research materials and assistance in lab work.

I appreciate Rivatex East Africa Ltd fraternity for enabling me do some research in the processing and textile laboratory as well as assisting in material needs and not forgetting all of my classmates for academic support and sharing ideas closely. May God Almighty reciprocate generously those who walked with me in the course of my study.

DEDICATION

I earnestly dedicate this thesis to my parents, Mr. Paul K. Tonui (post-humously) and Mrs. Sarah C. Tonui, my wife, children and siblings for their prayers, emotional and financial support during my study. Finally, most sincere gratitude to my colleagues, classmates and friends for their inspiring words, comfort, advice and encouragement.

ABSTRACT

Natural dyes unlike the toxic and carcinogenic synthetic have attracted global attention because of their biodegradability and non-hazardous nature. The present study sought to extract, characterize and evaluate the dye-ability beetroot (*Beta vulgaris)* peels and pomaces natural dye on cotton (cellulosic). This was made comparison to synthetic Reactive Orange HER with analyses of the dyed fabrics fastness properties. Extraction was performed by Soxhlet apparatus using methanol as solvent. Isolation using column chromatography and characterization by UV-Vis, FT-IR, HPLC-UV and LC-MS/MS were done. Betanin was identified by comparing its retention, *t*R, with betanin standard (betanidin 5-β-D-glucopyranoside) in the HPLC-UV and LC-MS/MS method. Minitab statistical approach, response surface methodology (RSM) and central composite design (CCD) were employed to design experiments aimed at optimizing chosen factors that affect extraction and dyeing namely; temperature (T), material to liquor ratio (M:L), time (t) and pH. Dyeing was performed by exhaust method, alongside mordanting. Pre, simultaneous and post mordanting techniques were employed while comparing the naturally obtained mordants (the order of a tannic acid-alum mordant combination) with synthetic mordants ($K_2Cr_2O_7$, $FeSO_4$ and $CuSO_4$). The CIELab and light, washing, rubbing and perspiration fastness of the dyed fabric were evaluated using standard methods. Reactive dyeing using Reactive Orange HER was done for colour equivalence (Ceq) and mass equivalent (Meq). The extraction optimized parameters of M: L ratio 1:10 (w/v) and t of 11 hours resulted in 40.68±1.45 % natural dye yield from the plant. The optimized parameters of dyeing were T of 55 °C, t of 75 minutes and pH 6. The UV-Vis spectrum of the extract and R. Orange HER showed peaks at wavelength of maximum absorbance (λ_{max}) at 538 and 492 nm respectively. The FT-IR of the isolated extract, operated in the range of 4000–500 cm^{-1} revealed absorption bands at 3282.82 cm^{-1} (O-H) for hydroxyl groups, 2932.96 cm^{-1}(C-H stretching) for alkane,1588.91 cm^{-1} (C=N) for amines,1342.83 cm^{-1} (C-H) aliphatic bending, 1045.38cm^{-1} (C-O-C linked symmetric stretching), 986.22 cm^{-1} (C-H bond deformation) and 918.87 cm^{-1} (C-COOH bond stretching). The FT-IR of dyed cotton fabrics displayed bands in 1025.23 and 985.28cm^{-1} attributed to deformation of C-H bonds probably due to mordant effects. The HPLC-UV and LC-MS/MS results confirmed the presence of betanin at retention time, *t*R of 7.699 and 7.71 minutes respectively, closely corresponding to *t*R (7.60 min) of the standard. The HPLC-UV estimated betanin content to be 3.81±0.31 mg/g. The main compounds revealed by LC-MS/MS data were; 2-*O*-glucosyl-betanin, betanin, isobetanin, betanidin, among others. The Ceq and Meq of the extract to the synthetic dye implied that 1 kg *B. vulgaris* peels and pomace extract is equivalent to 32.8±7.04 g kg^{-1} of synthetic dye. Colour fastness of the dyed cotton fabrics were in the range of 4-5 (very good to excellent). Post mordanting method in all the mordants gave the best colourfastness and colour strength (K/S) on the fabrics than other methods. The $FeSO_4$

and tannic acid-alum mordants yielded better results of K/S 3.60 and 3.92 respectively while $CuSO_4$ and $K_2Cr_2O_7$ yielded 3.60 and 1.20 respectively. These findings demonstrate that *B. vulgaris* peels and pomace extracts can be utilized to isolate the desired natural dye pigment (betanin) for coloration of cotton substrates with enhancement of natural mordants. Nevertheless, to achieve their maximum colour strength, there is need for further research on mechanisms to promote their chemisorption on fabrics.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ACRONYMS

AATCC: American Association of Textile Chemists and Colorists

ANOVA: Analysis of Variance

BC: Betacyanins

BX: Betaxanthins

CCD: Central Composite Design

Ceq: Colour equivalence

CI: Colour Index

CID: collision-induced dissociation

CIELAB: International Commission for Illumination

DART: Direct analysis in real time

DF: Dilution Factor

DOE: Design of Experiments

DOPA: dihydroxyphenylalanine

ESI: Electron spray ionization

ESTD: External standard

FTIR: Flourier Transform Infrared

GC-MS: Gas Chromatography –Mass Spectrometry

GSM: Grams per square metre

HPLC: High Performance Liquid Chromatgography

HPTLC: High Performance Thin Layer Chromatography

HTS: High-throughput screening

ISO: International Standards Organization

LC: Liquid chromatography

LFS: Light Fastness Standards

LOD: Limit of detection

LOQ: Limit of Quantification

MALDI: Matrix Assisted Laser Desorption Ionization

MBTL: Mercury Blinded Tungsten Lamp

Meq: Mass equivalence

MIR: Mid –Infrared

mL :Milliliter

MLR: Multiple Linear Regression

M.L.R: Material to liquor ratio

MS: Mass spectrometry

NMR: Nuclear Magnetic Resonance

o.w.f: on weight of fabric

ppm: Parts per million

P value: Significance value

PHWE: Pressurized hot water extraction

R_f: Retention factor

RSM: Response surface methodology

SAMDI: Self-assembled monolayers desorption/ionization

SDC: Society of dyers and colourists

SLS: Sodium Lauryl Sulphate

TLC: Thin layer chromatography

TOF: Time of flight

TRO: Turkey red oil

TSQ: Triple Quadrupole Mass Spectrometry

UHPLC: Ultra high performance liquid chromatography

UV-Vis: Ultraviolet visible

°C: Degrees celsius

ΔE : Relative change in colour/strength

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The appeal and aesthetic value of textile materials to the consumer is mainly due to colour(s) on them (Bhuiyan, A. Islam, S. Islam, Hossain, & Nahar, 2017). These colours are obtained either from dyes or from pigments (Yusuf, Mohammad, Shabbir, & Khan, 2016). Most dyes are water soluble in nature. Hence, aqueous dyeing method is mostly applied in dyeing of the textiles (Yusuf *et al.,* 2015).

Dyeing is an ancient art that existed way before discovery of history writing. Organic dyes have been the main color additives for many textile materials around the world (Frose, Schmidtke, Sukmann, Junger, & Ehrmann, 2018; Yusuf, Shabbir, & Mohammad, 2017). This practice can be traced back to the Bronze Age era in Europe. Europeans applied archaic dyeing technique involving sticking plants to fabric and rubbing crushed pigments onto clothes (Ado, Yahaya, Kwalli, Abdulkadir & 2014). In the entire period, dyes from natural sources were used secretively as they were regarded as the most beautiful and exotic pigments and this art was reserved for those in high status (Saxena, Tiwari, Pandey, 2012).

The introduction of man-made synthetic dyes has led to an almost complete displacement of natural dyes since most natural dyes exhibit poor to moderate wash and light fastness. Their synthetic counterparts represent the full range of color strength, wash and light fastness at relatively moderate costs (Stewart, 2017). Furthermore, they are found to have favorable application properties with a wide range of colors to select from, higher

reproducibility within a short time and improved quality of dyeing (Konar & Samantha, 2011). Still with the advent of synthetic dyes, natural dyes had an edge among the craft spinners, weavers, knitters and small scale level dyers who attracted small scale producers and exporters dealing with highly eco-friendly textile production (Saha & Sinha, 2012). The interest in the use of synthetic dyes has been reversed and in rapid decline due to the result of adverse environmental effects associated with them (Li, Xu, Tang, Zhang, & Mao, 2015). For instance, countries such as Germany have banned the use of azo dyes (Almahy & Ali, 2013). As a result, organic dyes are among the promising options for developing a greener textile dyeing process. This is evident by the increased number of recent research and publications such as Eco-driven" fashion, "sustainable clothing" or "green clothing" in the case of dyeing bacterial cellulose fibre (Costa, Rocha, & Sarubbo, 2017).

Natural dyes are non-pollutants becoming an ideal choice of dyeing due to their non-toxicity in nature, non-carcinogenic, easy handling and disposal since they are biodegradable (Shahid *et al.,* 2012). Natural dyes also contain bioactive compounds beneficial to our health. Many natural dyes have UV protective (Simpson, Hunter, & Aytug, 2015), antimicrobial (Wangatia, Tadesse, & Moyo, 2015), and deoxidizing (Baião, Silva, & Mere Del Aguila, 2017) properties. The shades produced by natural dyes are known to be soft, lustrous and soothing to the human eye (Sanjeeda and Taiyaba, 2014). They are obtained from renewable sources viz. plants, animals and minerals (Yusuf *et al*., 2017).

Beetroot (*Beta vulgaris* L. *ssp. vulgaris*) is a traditional vegetable widely distributed throughout the world. *B. vulgaris* is the main source for the organic red dye, the extracts

obtained normally referred to as “beetroot red”. Beetroot consist of multiple biologically active phytochemicals including betalains (Baião *et al.,* 2017) such as betacyanins and betaxanthins, flavonoids, polyphenols, Saponins (Baião *et al.,* 2017) and inorganic Nitrate (NO_3^-). Furthermore, it is a rich source of various minerals such as potassium, sodium, calcium, magnesium phosphorous, copper, iron, zinc and manganese (Singh & Hathan, 2014). Beetroot is commonly processed and consumed in form of juice, powder, bread, gel, boiled, oven-dried, pickled, pureed or jam-processed (Wruss *et al*., 2015; Morgado *et al*., 2016; Guldiken *et al.,* 2016).

Dyes have been applied on various common textile fibers including cotton, silk and cotton (cellulosic) fibre. Cotton is found throughout the world with many varieties and qualities. There are various types of cotton fabrics, among them unprocessed type like greige fabric. Greige cotton fabrics are first wet-processed through desizing, scouring, bleaching and mercerization before dying (Bhuiyan *et al.,* 2017). These pre-treatment processes before dyeing helps in absorption or improves dye uptake.

Synthetic dyes are toxic and non-environmentally friendly since they are produced from non-renewable and non-biodegradable petrochemicals (Li *et al*., 2015). Approximately 30 million tons of dyes are being consumed globally in textile industries with about 70,000 tons being released to the environment (Yusuf *et al.,* 2017). These disadvantages have necessitated attention towards the natural dyes as alternatives to synthetic dyes. To improve their performance characterstics, mordanting will be employed. It is for these reasons that this study was undertaken to utilize readily available raw materials which are regarded as wastes (peels and pomace) and hence lessening the dumping of organic wastes in the environment.

This study used beetroot peels and pomace to extract dye and apply on cotton fabrics, using natural and synthetic mordants for comparative purposes. In addition, this study established colour equivalence of the dye extract relating to synthetic dye of the same hue (Reactive orange HER, C.I. 84). This work evaluated dyeing properties of *B. vulgaris* for reasonable eco- colouration of cotton fabric as well as evaluate the optimum relative colour change percent, colour strength and colour fastness properties.

1.2 Statement of the Problem

Almost all the synthetic colourants are being prepared from petrochemical sources through chemical processes utilizing various harsh chemicals that are harmful to the environment. About 200, 000 tons of dyes are lost as effluents every year during the printing, dyeing and finishing processes probably due to inefficiency of printing or dyeing process or simply poor quality of dyes (Yusuf *et al.*, 2017). There is a worldwide concern over the carcinogenic effects, toxicity and allergic reactions associated with synthetic dyes. Natural dyes are an alternative to synthetic dyes for textile industries (Yamjala, Nainar, & Ramisetti, 2016) and toxic amines (Li *et al.,* 2015). People who are allergic and develop complications due to chemicals increases annually as indicated in the business week (Chaudhry *et al*., 2019; Arora, Agarwal, & Gupta, 2017). These effects have become common and hence the need to have a safer colorant that is environmentally friendly.

1.3 Main Objective

To characterize *B. vulgaris* peels and pomace extract and evaluate its colourimetric properties on cellulosic substrate while comparing it with synthetic dye.

1.4 Specific Objectives

Specific objectives were to:

i. Optimize extraction conditions and determine the extraction yield of *B. vulgaris* peels and pomace by solvent method.

ii. Characterize the extracts using UV-VIS and FT-IR spectroscopic techniques.

iii. Identify and quantify the natural dye extract using High Performance Liquid Chromatography (HPLC-UV) and Liquid Chromatography - mass spectrometry (LC-MS).

iv. Optimize dyeing process, analyze the fastness properties of the *B. vulgaris* peels and pomace dyed cellulosic substrates under different mordants and different mordanting methods, and comparative studies of natural vs metallic mordants as well as colour equivalence; natural dye vs Reactive Orange HER.

1.5 Research Questions

i. What is the extraction yield of *B. vulgaris* peels and pomace extract by solvent method?

ii. What are the phyto-chemical constituents in the *B. vulgaris* peels and pomace dye extract?

iii. What are the absorbance characteristics and chemical structure of *B. vulgaris* peels and pomace extract?

iv. How can the natural dye contents be efficiently isolated for identification and quantification?

v. How does the colour of the natural dye appear on cotton fabric after dyeing and by adding mordants; natural mordants comparing with metallic mordants, and in comparison to Reactive Orange HER?

1.6 Rationale/ Significance of the Study

Most synthetic dyes and metallic mordants are hazardous, have negative effect to human beings that include skin allergies and release vast amount of pollutants to the environment after manufacturing processes. They are toxic because of the raw materials and intermediate derivatives. Some synthetic dyes were synthezsied from heavy metals and azo groups, making them harmful to the environment and human health (Khan *et al.*, 2018a; Khan, Hussain, & Jiang, 2018b). Synthetic dyes are costly because of numerous artificial chemicals used to manufacture them. The dyes from *B. vulgaris* peels and pomace are human friendly since they have no carcinogenic or allergic effect for human beings especially for dyers and the environment. The raw materials used for the extraction of natural dyes are easily accessible in our surroundings at cheaper or low cost. Equally, it makes use of plant wastes such as peels and pomace. Natural dyes constitutes an essential part of the world's ecological and cultural heritage as their wide selection in colour production is beneficial to all generations. This brings value to the beetroot producers thus can improve their livelihood through dye production. The exploitation of *B. vulgaris* waste (peels and pomace) in this study will reinforce and increase knowledge in natural dyes through systematic reports.

1.7 Limitations of the Study

i. Some apparatus and equipment are inaccessible and are expensive.

ii. Time limitation to carry out exhaustive analysis.

iii. Lack of previous research studies in line with the specific topic.

1.8 Scope of the Study

Natural dyes from several sources are found to be very suitable for dyeing cotton or cellulosic fabrics. Demand is growing for such eco-friendly garment hence research or development of newer dye sources from new natural dyes should increase simultaneously. In this study, only one natural dye source was discussed, that is the red dye from *B. vulgaris* peels and pomace. For purposes of this research pure woven 100% cotton fabrics were used as the substrate due to the cellulosic components. They were easily available locally at Rivatex East Africa Ltd factory in Eldoret.

From previous studies, methanol is known for its high absorbance values and reportedly the best solvent for extraction of natural dyes (Manasa, Sindiri, & Vangalapati, 2013; Devi Rajeswari *et al.,* 2015) hence it was used as a solvent in this study. This study dwelt on natural ways of dyeing and therefore it entailed the use of natural mordants; order of tannic acid-alum combination. In comparing the natural mordanted sample, control samples included; un-mordanted samples and dyed samples pre-mordanted, simultaneously mordanted and post-mordanted with copper (II) sulphate ($CuSO_4$), potassium dichromate ($K_2Cr_2O_7$) and iron (II) sulphate ($FeSO_4$). This was an experimental study design and data was analyzed descriptively and inferentially. Various instruments of analysis were in use; column chromatography was used in fractionation (isolation) of compounds in the extract, Ultra violet-visible analysis (UV-VIS spectroscopy) and Fourier transform infrared (FT-IR) spectroscopy (on the crude extract and isolates respectively) for absorbance values and structure elucidation respectively. High pressure liquid chromatography (HPLC) and liquid chromatography–mass

spectrometry (LC-MS) were used in separation and characterization of the cleaned-up colorant molecules in the plant isolates. The fastness properties tests were determined using Launder-O-meter for washing fastness, Fadometer for light fastness, crockmeter for rubbing (dry and wet) fastness and perspirometer for perspiration (acid and alkali) fastness, following standard ISO methods guidelines. Their results were consequently analyzed using statistical techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1 Synthetic Dyes and Environmental Concerns

Up to late 19th century, people were using natural dyes obtained from plants for colouration of textiles (Yusuf *et al*., 2017). In plants, natural dyes are derived from roots, barks, leaves, fruits and flowers (Yusuf *et al*., 2015). After the discovery and invention of synthetic dyes, natural dyes ceased to be used because of the advantages of synthetic dye over natural dye with respect to application, colour range, robust fastness properties and availability (Stewart, 2017). However, most synthetic dyes have been realized to be hazardous, carcinogenic and release vast amount of pollutants to the environment. They are mostly derived from toxic raw materials and intermediate derivatives during their manufacturing process (Khan *et al*., 2018a, 2018b; Shahid, Islam, & Mohammad, 2013). The textile industry consumes hundreds of chemicals in production, hence regarded as the most polluting industry. The synthetic dyes used in textile wet processing produce effluents. They disturb the ecological balance and causes serious health issues as they contain high salt dosages and chemical auxiliaries (Ticha, Haddar, & Meksi, 2016). The effluents are not only damaging to the environment but also causing global warming and ozone layer depletion (Zheng, Fu, & Liu, 2011).

Textile dyeing and printing industries are facing sanctions due to hazardous impacts arising from the extensive utilization of synthetic dyes. As a result, the development of non-allergic, non-toxic and environment friendly natural dyes has attracted the attention of both researchers and industries (Shahid *et al.,* 2012; Rather *et al*., 2016a, 2016b; Uddin, 2015; Yusuf *et al*., 2016. Traders, consumers and suppliers in textiles are

nowadays analyzing the manufacturing, supply, application and the waste effects to the environment of various commercial dyes (Banerjee & Sharma, 2013).

The discovery of bladder tumors in various textile industry workers who were exposed to aromatic amines such as benzidine and beta-naphthylamine during azo dye manufacturing process became an eye-opener (Khan *et al*., 2018a, 2018b). This realization led to termination of the use of such adverse precursors in most of the western world countries. Azo dyes such as Direct Black 38 azo dye produces 22 different amines that are highly lethal that can undergo metabolism in mammalian systems to produce the benzidine precursor. The metabolism of these aromatic amines after being released from the azo dyes is a bother because of their potential to derive nitrenium ions that can react with DNA to produce mutations - an initial step in malignant tumor formation (Almahy & Ali, 2013). Figure 2.1 shows an aromatic compound undergoing azo cleavage resulting in production of free aromatic amine.

Step 1. The azo cleavage of aromatic azo bond to release free aromatic amine(s)

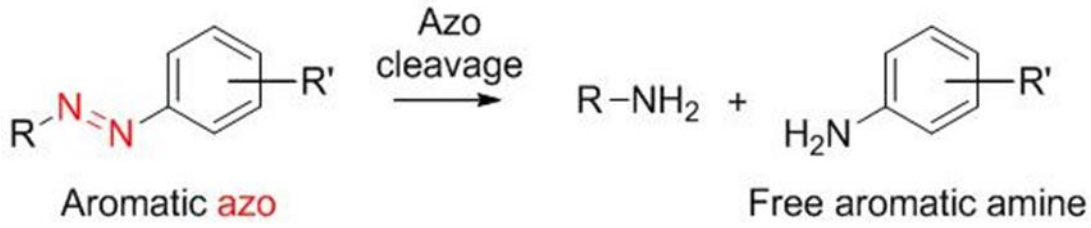


Figure 2.1. Cleavage of the azo bond to yield free aromatic amine (Almahy & Ali, 2013).

Regulations such as EU Directive 2002/ 61/EC were instituted immediately to prevent the sale of products containing dyes in the group aromatic amines that undergo an azo bond. The cleavage of the azo bond in the aromatic azo compound yields a primary

amine, denoted R–NH_2, and a free aromatic amine, denoted NH_2 bonded to a variably substituted aromatic system.

Step 2. Metabolic activation of the aromatic amine through nitrogen hyroxylation and oxygen acylation leading to formation of electrophilic reactants.

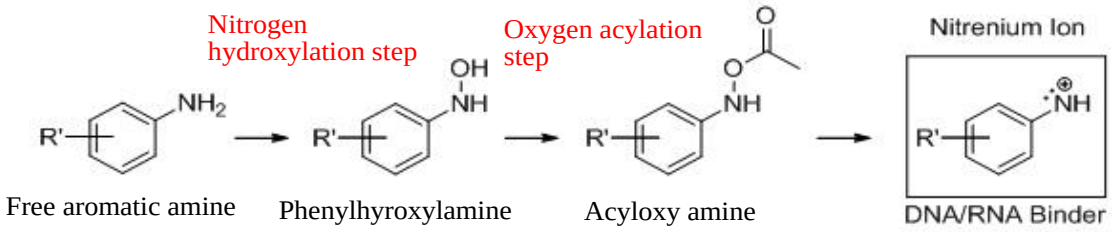


Figure 2.2. Metabolic activation of aniline to yield nitrenium ion (Almahy & Ali, 2013).

Figure 2.2 illustrates the step-wise metabolic activation of a variably substituted aniline, yielding an electrophilic nitrenium ion. Nitrogen hydroxylation first occurs on the variably substituted aniline, followed by acylation on the oxygen atom of the hydroxyl group consequently yielding an acyloxy amine. The last step involves removal of the acetate group in the amine to yield a nitrenium ion with an electropositive charge on nitrogen. The protonated nitrenium ion may readily bind covalently to DNA or RNA. These formation of aromatic amines by degradation of azo dyes has been shown to cause environmental pollution (Bru¨schweiler & Merlot, 2017; Rawat, Mishra, &Sharma, 2016).

2.2 Natural Dyes

Currently, ecological consideration is becoming an important factor in the selection of consumer goods all over the world especially in clothing or textiles. The adverse environmental impacts form synthetic dyes has created an increasing awareness and attention on research, development and use of natural dyes. Many researchers are

investigating the applicability of natural dyes in improving aspects of colouration such as affinity improvement to fabrics, colour coverage improvement and in special finishes like antitoxicity, and antimicrobial activity (Yusuf *et al.,* 2015). Because wastewater dyes have a complex structure and are very difficult to remove, this translates to different costly methods that are employed to eliminate them from the environment, whereas natural dye waste are biodegradable (Alsehri, Naushad, Ahamad, Alothman, & Aldalbahi, 2014).

Natural dyes are derived from plants, animals and minerals (Yusuf *et al.,* 2015). Natural dyes are mostly nonsubstantive and must be applied on textiles by the help of mordants as fixatives, usually a metallic salt, having an affinity for both the colouring matter and the textile fibre (Makkar, Singh, & Rose, 2013).

Dyes made from natural colours provide shades that are unique, soothing and friendly to the eye (Kusumawati, Santoso, Sianita, & Muslim, 2017). Colour hues that come up as different dye lots from the same plant extract can fade at different rates as they age (Yusuf *et al*., 2017). The positive attributes of natural dyes in addition to the energy saving and environmental safety of natural dyes have attracted the attention of many researchers and industrialists to develop natural dyes to be applied in textiles and other allied industries (Khan *et al*., 2014; Tutak & Benli, 2012). However, there are many vague areas that need to be researched on concerning natural dyes triggered by their limitations such as dyeing exhaustion and maximum absorption, mordant mixtures, etc.

2.2.1 Classification of Natural Dyes

The ancient way of classification was according to their order of alphabet or according to the botanical names. Much later, it was classified in diverse ways, for instance based on hue, chemical constitution and way of application (Konar & Samantha, 2011).

2.2.1.1 Classification Based on Application

Natural dyes are classified into two groups according to Prabhu and Bhute (2012); Group A constitutes 'Substantive Dyes' like indigo, turmeric etc. Group A dyes the fibers directly, while Group B constitute 'Adjective Dyes' such as logwood, madder etc. that are mordanted with a metallic salt for adherence onto the fibers (Rather *et al*., 2016b).

Group B natural dyes are classified as mordant dyes, vat dyes, direct dyes, acid dyes and basic dyes, and disperse dyes. Mordant dyes require a mordant in their application as they have no affinity to bind on the fiber directly (Rather *et al.*, 2016b). Group B dyes should have a mordant, that is an electron donating group capable of forming a complex with the dye .The mordant can be a transition metal salt. Example of such mordant dyes include madder, fustic, persian, berries, kermes, cochineal etc. Here three types of mordants can be used; metallic mordants such as metal salts of aluminum, copper, tin etc.; tannic mordant such as tannic acid, e.g., from myrobalan and sumach, and oil mordant that forms complex with main metal mordant (Ghouila, Meksi, Haddar, Mhenni, & Jannet, 2012).

Vat dyes are water insoluble dyes. During dyeing, vat dyes are first converted to their water-soluble form by reducing them with sodium hydrosulphite and then solubilizing it with alkali, and then applied on the fabrics. The true colour is produced only upon

oxidation followed by treatment with a solution of hot water and soap. An example here is indigo (Burkinshaw, Jeong, & Chun, 2013).

Direct dyes have tremendous affinity for the cellulosic fibers without any pretreatment of the dye or the fiber. Fabrics are dyed from a boiling dye bath. Examples include Turmeric, harda, pomegranate rind and annatto (Burkinshaw and Salihu, 2017).

Acid dyes are applied from an acidic medium and are most suitable for dyeing of wool and silk since they have either sulphonic or carboxylic group (s) which form electrovalent bonds with amino groups of wool or silk. Addition of tannic acid known as back tanning improves the fastness of these type of dyes, e.g., saffron (Clark, 2011).

Disperse dyes are applied onto hydrophobic synthetic fibres (such as polyester) in neutral to mild acidic pH medium (Clark, 2011). They can also be applied on silk and wool with the assistance of mordants such as chromium, copper and tin salts by post-mordanting method to fix them on fibres, e.g. lawsone or henna and many other flavones and anthraquinone dyes (Rather *et al.*, 2016b). Disperse dyes have low molecular mass and poor solubility due to their weak solubilizing groups.

Basic (cationic dyes) when in ionized form yield coloured cations and form an electrovalent bond with the –COOH group of wool and silk fibre. Basic dyes work well in neutral to mild acidic pH. These dyes have poor light fastness e.g. berberine (Burkinshaw *et al.*, 2013).

2.2.1.2 Classification Basing on Hue or Colour

Group B natural dyes that are categorized according to hue as stipulated by Colour Index (C. I.) International (Konar & Samantha, 2011), a database jointly maintained by Society

of Dyers and Colourists (SDC) and American Association of Textile Chemists and Colorists (AATCC). Red colour dyes mostly red natural pigments are stored in roots or barks of plants or camouflaged in the body organs of dull grey insects. Red colour natural dyes are mostly anthraquinone and its derivatives. These dyes display great stability to light and washing. The CI lists 32 red natural dyes and the few prominent members in this category are manjistha (*Rubiacordifolia*), madder (*Rubiatinctorum*), Brazil wood/sappanwood (*Caesalpineasappan*), Al or morinda (*Morindacitrifolia*), cochineal (*Coccuscacti*) and lac dye (*Coccuslacca*) (Yusuf *et al.*, 2017).

Yellow colour dyes are the most common and abundant of all hues in nature. Yellow colour natural dyes are 28 in number from the Colour Index data. Majority of the yellow dyes are flavonoids produce pale shade with faster fading except turmeric, which exhibit dull deep shade. Wash fastness of natural yellow dyes rates from fair to excellent, e.g. tesu flowers (*Buteamonosperma*), barberry (*Berberisaristata*), turmeric, kapila and kamela (*Mallotusphilippensis*) (Yusuf *et al.*, 2017).

Blue colour as Indigo and woad give excellent colour fastness to light and washing. According to the CI list, only four blue natural dyes have been recognized and they appear as; natural indigo, sulphonated natural indigo, Kumbh (*Manipur*) and the flowers of Japanese known as ‘Tsuykusa’ that are used mainly for making awobana paper (Sinha, Saha, & Datta, 2012). Table 2.1 shows the number of natural dyes in each hue as per the Colour Index (Konar & Samantha, 2011).

Table 2.1. Number of natural dyes and percentage in each hue as per the Colour Index.

CI Natural	No. of Dyes	Percent
Yellow	28	30.4
Orange	6	6.50
Red	32	34.8
Blue	3	3.30
Green	5	5.50
Brown	12	13.0
Black	6	6.50

Black and Brown colour dyes give black or dark brown shades, and are generally obtained from tannin rich plants such as wattle, logwood, harda, custard apple, goalnuts, lac, carbon, caramel and roots of iris plant (Yusuf *et al*., 2017). Natural brown colours have no limit in terms of its natural sources. Cutch dye for example is a brown dye extracted from wood of acacia trees (e.g. acacia catechu) and has been in existence since ancient times in dyeing cotton to achieve shades of brown. According to Table 2.1, the CI of black natural colour has six dyes. These dyes are compatible towards cellulosic and protein fibre, and imparts good overall fastness properties (Yusuf *et al*., 2015, Yusuf *et al.*, 2017).

Green dyes from natural sources are rare. Both woad (*Isatistinctoria*) and indigo are used in combination with yellow dyes to produce green shades. Soft olive green shades are also obtained when yellow dyes are applied on textile and treated with iron mordant (Konar & Samantha, 2011).

Orange dyes can be obtained from sources that create reds and yellows. Some sources of natural orange dyes are annatto, barberry, sweet pepper bloodroots etc. (Tayade & Adivarekar, 2013).

2.2.1.3 Classification Based on Origin

Based on their sources of origin, natural dyes are vastly classified into vegetable, mineral and animal origin dyes. There are approximately five hundred (500) vegetable origin dyes, whereby the colouring matter is derived from root parts, leaf, bark, trunk or fruit of plants as detailed in the Table 2.2 (Yusuf *et al*., 2017, Sinha *et al*., 2012).

Table 2.2 Common vegetable dyes.

Plant part	Dyestuff
Flowers/Petals	Dahlia, Tesu, Marigold, Kusum.
Fruits/Seeds	Pomegranate rind, Latkan, Myrobolan (Harda), Beetle nut.
Leaf	Cardamon, Tea, Coral Jasmine, Eucalyptus, Lemon Grass, Henna
Bark/Branches	Sappan wood, Red Purple bark, Sandal wood, Shillicorai, Khair
Root	Beet-root ,A. cepa, Turmeric

Animals also provide natural dyes and are extracted from dried body of insects like cochineal and lac. The chemical structures, botanical names and other specifications of a few popular animal natural dyes are provided in the C. I. (Adeel, Bhatti, Kausar, & Osman, 2012; Yusuf *et al*., 2015; Yusuf *et al*., 2017).

Mineral dyes or mineral colours originate from specific mineral natural source or derived from purified inorganic compounds. Some of the important and known mineral dyes are chrome yellow, ironbuff, manganese brown, narkin-yellow and Prussian blue. Mineral dyes from animal origin are lac, cochineal and kermes mostly derived from the insects (Yusuf *et al*., 2015, Yusuf *et al*., 2017).

2.2.1.4 Classification Based on Chemical Constitution

Based on their chemical constituents, it includes mainly indigoid dyes, alphanaphthoquinones, anthraquinone, flavonoids, di-hydropyrans, carotenoids and anthocyanidins (Konar & Samantha, 2011).

Alphanaphthoquinones are best known in India since about 4000 years as α-naphthoquinone dyes (Yusuf & Mohammed, 2017). The most prominent member in this class is lawsone or henna that is obtained from *Lawsonia inermis*. It is mainly cultivated in India and Egypt (Yusuf *et al*., 2017).

Flavonoids yield yellow dyes, and are classified under flavones, isoflavones, aurones and chalcones. Flavones are colourless organic compounds. Most natural yellow colours are derived from hydroxyl or methoxyl substituted flavones and isoflavones e.g. weld (containing luteolin pigment). They give brilliant and fast colours on both wool and silk (Yusuf *et al*., 2017).

Flavonols and anthocyanins phytochemicals constitutes the main subclasses of flavonoids present in plants. Anthocyanins are found mainly in red onions (Basak *et al*., 2012; Kopsell *et al*., 2010) and can produce a variety of colours such as orange, red maroon and blue.

2.2.1.5 Based on Chemical Structure

This is a widely accepted and the most appropriate system of classification. This is because the chemical structure readily identifies dyes belonging to a particular chemical group in which the structure determines certain characteristics. Examples include

berberine yielding dye plants such as *Berberis aristata sp., Berberis vulgaris sp., Rhizoma coptidis sp.,* etc (Khan, Harsha, Giridhar, & Ravishankar, 2012).

Carotenoids also called tetraterpenoids are brightly coloured natural organic pigments found in the chromoplast of plants. Quinonoids are widely distributed in nature and their colour ranges from yellow to red. Chemical structures of naturally occurring quinones are varied and more diverse than any other group of pigment yielding plants. Quinones based on their chemical structures are further classified as benzoquinones, α-naphthoquinones and anthraquinones. Gaudich *(Choloraphora tinctoria),* Cochineal *(Dactylopius coccus),* Henna or Mehendi *(Lawsonia inermis/L. alba),* Safflower *(Carthamus tinctorius),* Sundew *(Drosera whittakeri)* and Walnut *(Juglans regia)* are some of the natural resources for quinonoids class; subclass anthraquinonoids and naphthoquinonoids (Yusuf *et al*., 2016, 2017; Khan *et al*., 2012).

Flavonoids dyes have various plant sources that include Carajuru/Puca (*Bignonia chica/Arrabidaea china),* Flame of the forest or Palas (*Butea monosperma/Butea frondosa),* Hemp *(Datisca cannabina),* Jackfruit (*Artocarpus heterophyllus/Artocarpus integrifolia),* Onion *(Allium cepa),* Kaiphal (*Myrica esculenta),* Kamala *(Mallotus philippinensis),* Red Sandalwood *(Pterocarpus santalinus)* and Weld (*Reseda luteola)* (Khan *et al*., 2012).

Betalains can also be classified structurally. Beetroot are the common natural sources for betalains class of colorants (Ali & El-Mohamedy, 2011).

Tannins are obtained from the various parts of the plants such as fruit, pods, plant galls, leaves, bark, wood, and roots. These dyes are usually classified into two groups;

hydrolysable (pyrogallol) and condensed tannins (proanthocyanidins). The hydrolysable tannins are polyesters of a sugar moiety and organic acids, grouped as gallotannis and ellagitannins which on hydrolysis yield galllic acid and ellagic acid, respectively (Shahid *et al*., 2012). Tannins are largely used in glues, inks, surface water treatment to remove heavy metals and mordanting (mixing with other natural dyes to improve the affinity of fibres as well as providing different shades like yellow, brown, grey and black). Some plant sources rich in tannins include Cutch *(Acacia catechu)*, Gallnut *(Quercus infectoria)*, Harda *(Terminalia chebula)* and Pomegranate or Anar (*Punica granatum)* (Shahid *et al*., 2012).

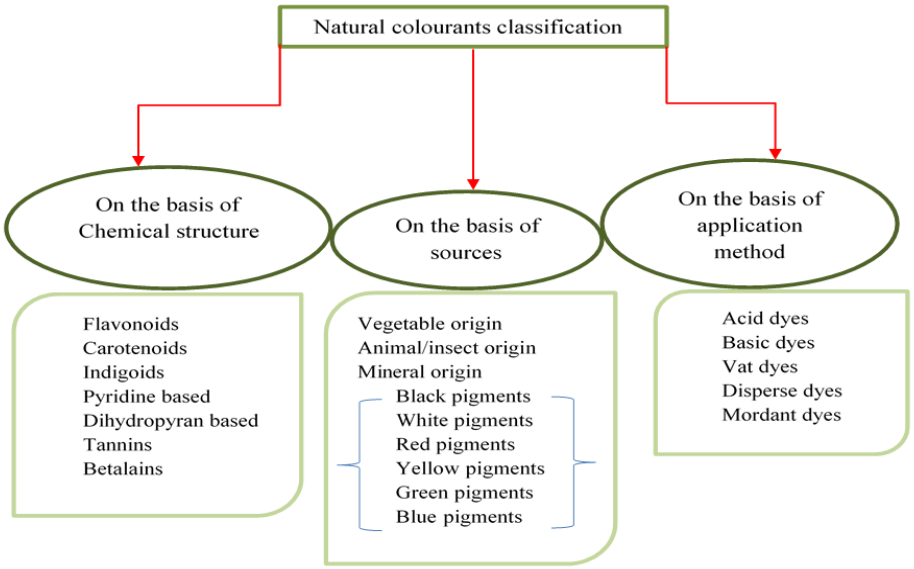


Figure 2.3. Classification chart for natural colourants (Konar & Samantha, 2011).

2.2.2 Advantages and Disadvantages of Natural Dyes

In the recent years, there has been a trend to revive the art of natural dyeing using organic dyes. This is mainly because natural dyes in most cases have more advantages than synthetic dyes. The shades produced by natural dyes are known to be soft, harmonizing, lustrous and soothing to the human eye (Konar & Samantha, 2011; Sanjeeda & Taiyaba, 2014). Natural dyes produce a wide variety of colours by mix and colour match system. A small variation in the dyeing technique and change in mordants with the same dye can shift the colours to a wide range of depth or may create totally new colours, a property not easily achievable with synthetic dyestuffs. Natural dyes are obtained from renewable sources that also contain bioactive compounds beneficial to our health such as antimicrobial activity hence considered as non-carcinogenic and non-toxic for human health (Ghoulia *et al*., 2012, Khan *et al*., 2012, Yusuf *et al*., 2015). They are also insect repellent (Almahy & Ali, 2013), and have fluorescence and UV protection properties (Rather, Islam, & Mohammad, 2015); Sun & Tang, 2011). Some natural dyes can be extracted from industrial by- products or from plant waste such as peels or pomace hence fit the zero emission approach (Konar & Samantha, 2011). There are no disposal problems in natural dyes since in most cases they find other uses, such as manure in agricultural fields. Natural dyes are usually renewable, biodegradable and are agro-renewable vegetable based just like *B. vulgaris* that is a vegetable (Yusuf *et al*., 2015).

However, natural dyes do have some disadvantages that are associated with the decline of this ancient art of dyeing textile. It may not be possible to reproduce shades by using natural colourants due to varied plant seasons, locations, species and maturity period. Thus, it is quite difficult to optimize and standardize a recipe of natural dyes for particular

plant(s), as the natural dyeing process depends on colour component and materials in use (Arora *et al.,* 2017).

Natural dyeing requires skilled workmanship hence it is expensive. This is also aggravated with poor colour yield of some sources of natural dyes leading to more usage of dyestuffs, increased dyeing time and excess cost for mordants. Therefore, research in new natural dyes sources along with eco-friendly, robust and cost-effective technologies for their extraction and application to widen the scope of natural dyes is vehemently needed (Shahid *et al*., 2013).

The dyed textile using natural dyes may change colour when exposed to sun, sweat and air for long periods of time. Thus, with a few exceptions, all natural dyes require the use of mordants to fix them on the textile substrate. However, surface excess may occur such that a significant percent of the unexhausted mordant may remain in the residual dye bath and as such may pose serious effluent disposal problem. With a few exceptions, most of the natural dyes may not respond well in colour adherence when applied in conjunction with a mordant. Therefore, sometimes their colourfastness performance ratings are inadequate for modern textile usage (Konar & Samantha, 2011).

2.3 *Beta vulgaris* Root

The beetroot is the tap-root part of the beet plant. Other names alluded to the plant includes; the garden beet, golden beet, red beet, or table beet (Figure 2.4). The cultivated varieties of *B. vulgaris* are grown for their leaves and taproots that are edible. Other than as a food, beets have been utilized as a medicinal plant and in food colouring. It is a

flowering and true biennial hence rarely perennial plant. It constitutes a variety with bulk colors ranging from yellow to red (Gokhale & Lele, 2014).

Beets were cultivated prior to the tenth century according to European written records (Yashwant, 2015). Several varieties of beetroot for example yellow beets emerged from 1700s and sugar beets were majorly cultivated by Prussians in the 1800s (Chawla *et al*., 2016). Red beets are currently popular and its genesis is linked to the Mediterranean region. The plant is well spread and widely cultivated in Europe, America and throughout Asia (Chawla, Parle, Sharma, & Yadav, 2016; Zohary, Hopf, & Wesis, 2012). In Kenya, it is grown in small scale and mostly varieties such as cylindra, bolt hardy, burpee's golden and chioggia pink.

Beets can be grown throughout the year and can tolerate to high temperature, optimum temperature ranging between 15° to 19° C and soil pH of 6-7. The lower temperatures favour the rapid growth of beets and promotes the development of their deep red pigmentation (Yashwant, 2015). The nitrogen availability affects the overall sugar content of beetroot hence it is advisable to apply in the early stages of growth. Harvesting and its quantity of yield depends on the fertilization, climate, disease infestation and variety of the plant (Yashwant, 2015).

Figure 2.4. *Beta vulgaris* root.

2.3.1 Taxonomy and Cultonomy

B. vulgaris is an economically important crop of the large order caryophyllales. Three subspecies are distinctly recognized. All cultivars in the cultonomy of the plant fall into the subspecies *B. vulgaris* subsp. *vulgaris*. The wild species ancestor of the beets is the sea beet (*B. vulgaris* subsp. *maritima*) (Chawla *et al*., 2016). This Plantae belongs to the subkingdom Tracheobionta - vascular plants, superdivision – Spermatophyta - seed plants, division - Magnoliophyta - flowering plants, class - Magnoliopsida - dicotyledons, subclass - caryophyllidae, order - Caryophyllales, family - Chenopodiaceae - Goosefoot family, genus- *Beta* L. - beet ,species - *Beta vulgaris* L. - Common beet. Chenopodiaceae family boasts of approximately 1400 species divided into 105 genera (Chawla *et al*., 2016) and also the members of dicotyledonous family. *B. vulgaris* is included among the plants in Betoideae subfamily in the Amaranthaceae family.

2.3.2 Phytochemical Constituents of *Beta vulgaris* Root

Among the numerous natural dyes, *B. vulgaris* peels and pomace are promising sustainable agricultural bio waste dye source. *B. vulgaris* is dark purple in physical

appearance and a distinctive purple flesh. Beetroot is a root vegetable with carotenoids, ascorbic acid, flavonoids, saponins, vitamins, minerals for instance sodium, calcium, potassium, phosphorous, magnesium, copper, iron, zinc, manganese and high levels of nitrate (644 to 1800 mg/kg) (Clifford *et al*., 2015; Guldiken *et al*., 2016; Lidder & Webb, 2013; Mikolajczyk-Bator, Blaszczyk, Czyżniejewski, & Kachlicki, 2016). From different research information, *B. vulgaris* also contains highly active colouring water soluble pigments betalains such as betacyanins (red-violet color) and betaxanthins (yellow-orange color) (Kumar & Su-Ling Brooks, 2018). All the compounds present in *B. vulgaris* have numerous nutritional and health benefits (Panghal *et al.*, 2017). This has led to conclusion by many researchers that beetroot is an important source of health promoting phytochemicals (Clifford, Howatson, Wes, & Stevenson, 2015) due to their antioxidative, anti-inflammatory, anticarcinogenic, antimicrobial and antiviral activity. They also possess other health benefits such as anti-diabetic, cardiovascular disease lowering, hypertensive and wound healing benefits. A few bioactive compounds are found in *B. vulgaris* in small quantities for example betaine, glycine and folate as identified by researchers (Clifford *et al*., 2015).

2.4 Betalains

Betanins or betalains are active alkaloid nitrogenous pigments that are water-soluble. The main component of *B. vulgaris* extract is betanin which are natural pigments known for its red color (Kathiravan, Nadanasabapathi, & Kumar, 2014). Betanin belongs to a group of molecules known as betalains (Gokhale & Lele, 2014). Red *B. vulgaris* constitutes the richest source of betalains, a group of pigments that contains the red pigments (Betacyanins) and yellow pigments (Betaxanthins) (Esatbeyoglu, Wagner,

Schini-Kerth, & Rimbach, 2015). The two betalains compounds are distinct basing on their chemical structures and compositions. (Slatnar, Stampar, Vebaric, & Jakopic, 2015) identified the presence of fifteen compounds from betacyanin group and seven compounds from betaxanthin group in only two red beetroot varieties. The same study elaborated betalain profile only in three parts of red beetroot. But Lee, An, Nguyen, Patil, Kim, & Yoo, 2014) described the composition of betalains in nine beetroot cultivars, but pointed out only four betalain compounds.

Betalains are derivatives of betalamic acid synthesized from the amino acid tyrosine into two structurally different groups classified as the red-violet betacyanins (BC) and the yellow betaxanthins (BX) (Slatnar *et al*., 2015). BC and BX differ by conjugation of a substituted aromatic nucleus to the 1, 7-diazaheptamethinium chromophore present in betacyanin. Other compounds that contribute towards the coloration of beets include vulgaxanthin and indicaxanthin. The varieties and depth of red-purple colour among beets depend on the ratio of betacyanin and betaxanthins present in that particular species (Szopinska & Gawęda, 2013). Betaxanthin consists of two types; vulgaxanthin-I and vulgaxanthin-II (Ravichandran *et al*., 2013). Several betacyanins are found in peels of beetroots for instance betanin, prebetanin, isobetanin, and neobetanin (Nemzer *et al*., 2011). Betalain has a molecular formula; $C_{24}H_{26}N_2O_{13}$ and its Molecular Weight is 550.473 g/mol. Previous studies have indicated that betacyanins and betaxanthins are capable of absorbing visible light. Due to their structural distinction between the two compounds, they have different light absorbing capacity (Figure 2.5).

Tyrosine is the genesis of betalain synthesis process. The whole betalain biosynthesis process involves two key enzymes tyrosinase and dihydroxyphenylalanine (DOPA).

Tyrosinase hydroxylates tyrosine resulting in massive accumulation of the later and produces DOPA. DOPA is converted to the *cyclo*-DOPA by the diphenol/DOPA oxidase activity of tyrosinase catalysis and its cleavage of the aromatic ring yields betalaimic acid (Hatlestad *et al*., 2012). Betacyanin is then formed by condensation of *cyclo*-DOPA with betalamic acid while betaxanthin is generated by condensation of an amino acid or amine with betalamic acid.

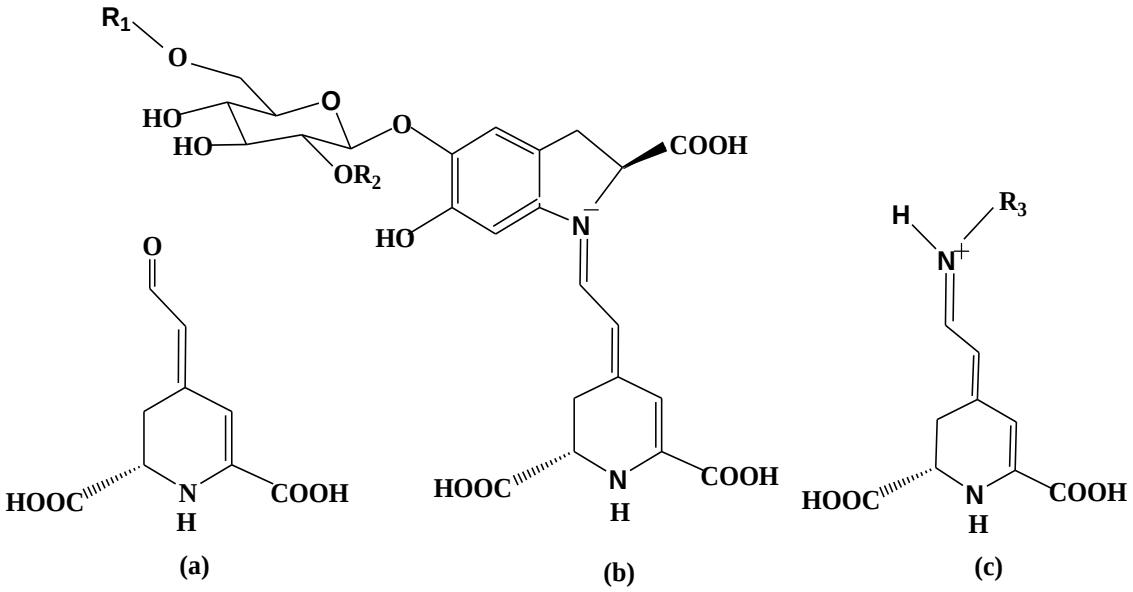


Figure 2.5. General structures of betalamic acid derivatives; betacyanins (a), betaxanthins (b) and Betanin (c): $R_1 = R_2 = H$. R_3 = amine or amino acid group (Azeredo, 2009).

2.5 Phenolic Compounds

The flavonoids are the biologically active phenolic compounds with good antioxidant activity (Chhikara *et al*., 2018). According to Vulic *et al*. (2014), the main types of flavonoids in *B. vulgaris* are betavulgarin, betagarin, cochliophilin A and dihydroisorhamnetinas. Several flavanones have ever been isolated from *B. vulgaris*, including the betagarin (5, 2-dimethoxy-6, and 7-methylenedioxyflavanone),

betavulgarin (2′-hydroxy-5-methoxy-6, 7- methylenedioxyisoflavone), 3, 5- dihydroxy-6, 7-methylenedioxyflavanone, 5-hydroxy-6, 7-methylenedioxyflavone, 2, 5-dihydroxy-6, and 7-methylenedioxyisoflavone. The ethyl acetate extraction of the flavanones reported are quercetin, rutin and 4′-hydroxy-5-methoxy-6, 7-methylenedioxy flavanone (Maraie, Abdul-Jalil, Alhamdany, & Janabi, 2014).

Some of the highly unstable phenolic compounds to have been isolated from the red beetroot are 5, 50, 6, 60-tetrahydroxy-3, 30-biindolyl; a dimer of 5,6-dihydroxyindolecarboxylic acid and betalains comprising of the stated vulgaxanthin I, vulgaxanthin II, indicaxanthin, prebetanin, isobetanin, betanin and neobetanin (Nemzer *et al.,* 2011). Moreover, two phenolic amides *N-trans*-feruloyltyramine and *N-trans*-feruloylhomovanillylamine have been extracted from the seed wall of beetroot (Nemzer *et al.,* 2011). Some *B. vulgaris sp.* (var. *cicla*) are reported to contain a significant quantity of hydroxybenzoic and hydroxycinnamic acid derivatives, which are the two major classes of phenolic acids subdividing into ten types. The phenolic acids constitutes catechin hydrate, epicatechin, rutin, protocatechuic, vanillic, *p*-coumaric, caffeic acid, proline, syringic acids and the monoterpenedehydrovomifoliol (Maraie *et al*., 2014). Figure 2.6 provides a summary of bioactive compounds present in *B. vulgaris* according to Ninfali & Angelino (2013).

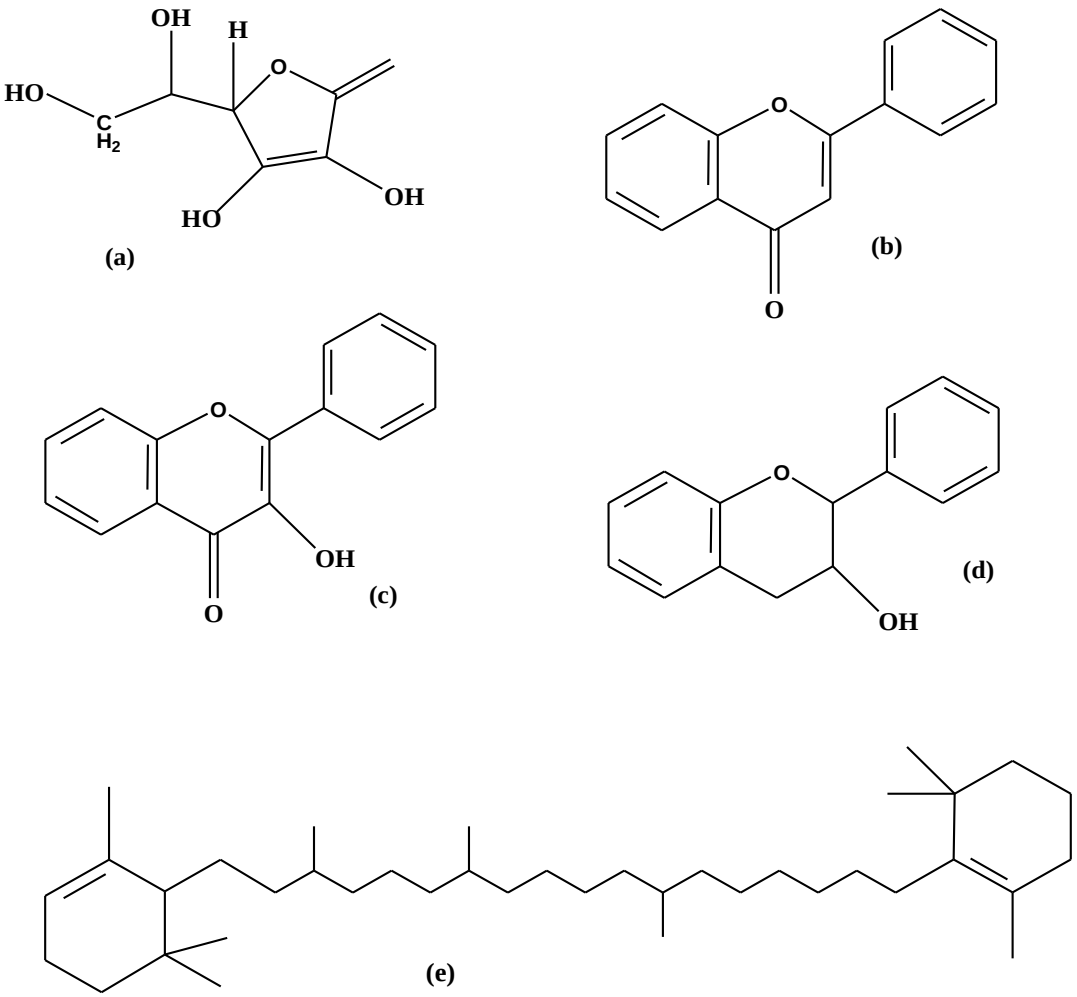


Figure 2.6. Structures of some bioactive compounds in *B. vulgaris* (data from Ninfali & Angelino, 2013); (a) Ascorbic acid, (b) flavone, (c) flavonol, (d) flavanol and (e) α-carotene. Other bioactive compounds in *B. vulgaris* plant are presented in Figure 2.5.

2.6 Carotenoids

The carotenoids present in *B. vulgaris* are a group of phytochemicals playing a pivotal role in the prevention of diseases acting as anticarcinogens, antioxidant and immuno-enhancers. Carotenoids have been reported to inhibit mutagenesis, an activity leading to decreased risks of cancer (Sardana, Chhikara, Tanwar, & Panghal, 2018). *B. vulgaris* leaves contain β-carotene and oxygenated derivatives known as xanthophyll such as

lutein. Rebecca, Sharmila, Das, & Seshiah (2014) reported that 1.9 mg/100 g of carotene are present in beetroot.

2.7 Saponins

B. vulgaris are also reported to contain saponins phytoactive compounds. They are produced by plants for defensive mechanism i.e. to counteract pathogens. Among the identified triterpene saponins in the *B. vulagris* from different studies include oleanolic acid derivatives. From its root extracts, betavulgarosides I, II, III, IV, V, VI, VII, VIII saponins have been discovered whereas betavulgarosides I, II, III, IV, V, IX, and X saponins are traced to the leaves according to Mroczek, Kapusta, Janda, & Janiszowska (2012). Furthermore, Mikolajczyk-Bator & Pawlak (2016) have also reported twenty six triterpene saponins characterized in *B. vulgaris*. Among these, seventeen triterpene saponins had not been previously reported while seven were rated as new compounds. Mroczek *et al*., 2012 identified the saponins using reverse phase liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI/MS/MS). Oleanoic acid is of great importance among the saponins identified in *B. vulgaris* peels and pomace as it is capable of causing a marked hypoglycemic effect.

2.8 Extraction Methods of Natural Dyes

Extraction can be defined as the separation of the desired components of a material source by physical or chemical means, with the aid of a solvent or aqueous media (Konar & Samantha, 2011). Natural dyes can be extracted using different methods such as; aqueous method, alcoholic or organic solvent extraction, enzyme assisted extraction, supercritical fluid extraction and soxhlet extraction method using either alcohol or benzene. Solvent extraction is the most preffered method. Phytochemicals are more

soluble in solvents, and causes aggregation of molecules such as dyes or pigments in plants (Zhou, Wu, Gao, & Ma, 2011). Aggregation of dye molecules is strongly dependent on the properties of solvents such as polarity, acidity, dye combination and temperature (Lim, Damit, & Ekanayak, 2015; Warkkoyo, 2011). Therefore, you can select the type(s) of solvent(s) basing on stated reasons for optimum yields and efficiency. For instance, polar molecules have been extracted using the most common solvents; ethanol, methanol, acetone etc. (Warkkoyo, 2011; Zhou *et al*., 2011). The process of extracting phytochemicals in natural products is a sequence involving; the plant absorbing the solvent into its solid matrix mass; the phytochemicals (solute) dissolves in the solvents; the solute is diffused out of the solid matrix differentially; and finally the extracted solutes are collected for further processes (Li *et al*., 2014) such as filtration, concentration through evaporation and drying using methods such as ultra filtration equipment, centrifuge rotary vacuum pump or extraction under reduced pressure to obtain a solidified sample.

The common solvents used are water, hexane, ethyl acetate, ether, benzene, chloroform, methanol and ethanol. Solvents are commonly used in combination with other technologies such as solidification or stabilization, precipitation and electro spinning (Li *et al*., 2014). Extraction of bioactive compounds is influenced by various process parameters such as solvent composition, pH, temperature, extraction time and solid to liquid ratio (Li *et al*., 2014). However, optimum conditions of extraction can be determined by varying extraction parameters of the colourant and then measuring the optical density of the corresponding extracts using a spectrophotometer.

2.8.1 Extracting Using Aqueous Solution

This is a conventional method of extracting natural dyes from diverse natural sources. The main medium of extraction is water with or without addition of salt, acid, alcohol or alkaline. The wet pretreatment of fabric such as cold bleaching, scouring, hot bleaching and mercerization to increase the dye absorbibity can cause pollution to the environment and consume a lot of water (Khandegar & Saroha, 2013). For optimal extraction conditions, the finely cut material or plant part is dissolved in measured quantity of water and extraction carried out under varying conditions such as extraction temperature, time, pH, material to liquid ratio, concentration of salt and mordant. In each case, the optical density or absorbance value (maximum) of the aqueous extract can be analysed using UV-VIS spectrophotometer (Konar & Samantha, 2011).

The common experimental procedure in aqueous extraction under normal conditions is boiling dried or fresh samples from low temperatures to the boiling point of water, cooling the filtrate, filtering and storing it for further analysis or drying after filtration into powder form for optimization purposes. In optimization, the extraction process of dye liquor is carried out under varying condition such as extraction time, extraction temperature, pH of extraction liquor, concentration of colour/source material and MLR (Sengupta, Mondal, & Mukherjee, 2015). Water is assumed an inevitable solvent since it is not harmful and due to its polarity, it preferentially extracts polar compounds such as plant pigments and tannins.

2.8.2 Solvent Extraction

Flavonoids and anthocyanins are soluble in polar solvents while the glucosides and aglycones are more soluble in water and alcohols respectively. Literature relating to the

extraction of natural dyes using solvent extraction technique includes Devi-Rajeswari *et al.* (2015) who used Mexican marigold flower with different solvents such as methanol, ethanol, hexane and water in investigating its dyeing effects on different fabrics and yarn samples.

Among the literature relating to solvent extraction of natural dyes includes that of Manasa *et al*. (2013) who used ethanol and methanol at 50:50 percent ratio for the extraction of natural dye from onion skin powder. Whatman No. 1 filter paper was used to filter the extracts and thereafter physico-chemical parameters present in the dye were estimated. In another study involving extraction of quercetin, rutin and total phenolic content, it established that methanol gave the best results (Manasa *et al.,* 2013). During the extraction of anthraquinones from the roots of *morinda citrifolia,* pressurized hot water extraction (PHWE) method at varied temperatures of 100, 170 and 220 °C was used. From the study, the extraction yield increased with increase in temperature resulting into increased solubility. In the extraction of eco-friendly natural dye from the stems of *Achras sapota* and flowers of *Spathodea campanulata* ethanol was used and dyeing properties in respect to colour strength and fastness properties of the two compared. The research found that a dye was present in both the stem and flowers (Kumaresan, Palanisamy, & Kumar, 2011) of the respective plants in the study.

Soxhlet extraction technique with solvents is currently applicable in most research works. This method involves discontinuous extraction process in that the extraction solvent inside the boiling flask undergoes evaporation and re-condensation inside the distillation column. The solvent then drops onto the solid material under extraction. The chamber containing the solid material in a thimble is connected to the boiling flask below

by a syphoning mechanism (like that of Pythagorean cup) that allows the chamber to fill to a point, at which it will empty its contents and start to fill again and the extracted compounds will accumulate in the boiling flask below the apparatus.

2.8.3 Supercritical Fluid Extraction (SCFE) Technique

Due to the rigorous environmental regulations and requirements in recent years, supercritical fluid extraction (SCFE) technique has gained wide acceptance as an alternative to conventional solvent extraction. It is mainly employed in separation of organic compounds in many industrial processes, analytical procedures such as extraction of organic compounds from herbs and natural colourant from plants. This method is among the advanced extraction techniques and involves the use of supercritical fluids as the extracting solvent acting on a solid matrix, but can also be from liquids. Carbon dioxide (CO_2) gas is the most regularly used supercritical fluid. The gas is sometimes modified with co-solvents (ethanol or methanol) with the extraction conditions above the critical temperature of 31 °C and critical pressure of 74 bars. The conditions that can be altered by addition of modifiers. A case example is the extraction of natural colorant carotenoid dye that gave high yield when compressed CO_2 gas was applied (Kulkarni, Gokhale, Bodake, & Pathade, 2011). In this method, the extract is dissolved with gas and converted into dissolved dye that is then absorbed on fabric effectively and the gas is recovered after dyeing process.

2.8.4 Enzyme-assisted Extraction Technique

This method makes use of specific enzymes to disrupt the cell outer components of the material under extraction. In combination with various techniques, this technique can be

efficient in overall recovery of bio-actives in a natural material. Figure 2.7 provides an overview of extraction procedures of natural colorants.

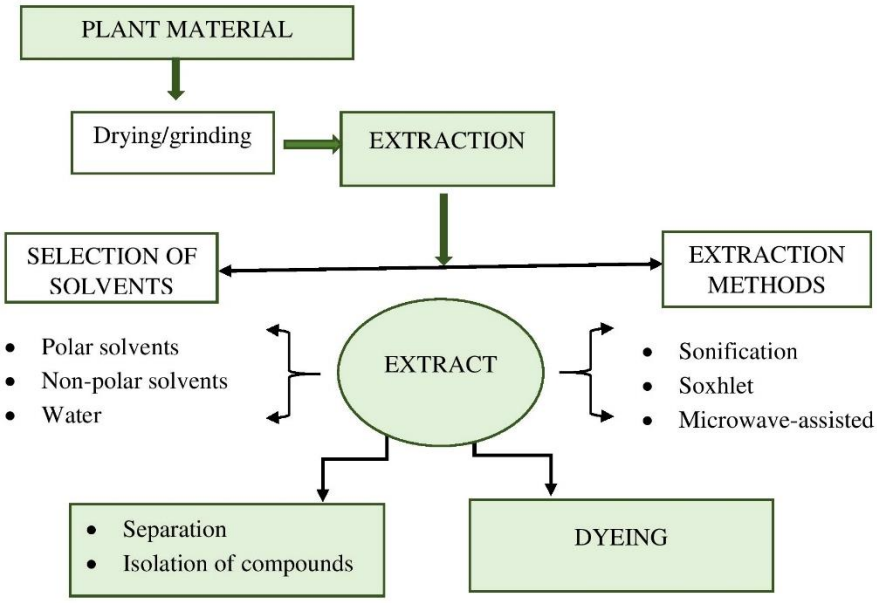


Figure 2.7. Schematic representation for extraction of natural colourants (Konar & Samantha, 2011).

2.9 Isolation, Identification and Characterization of Phytochemicals

A number of different techniques of separation are employed in isolation, identification and characterization of the bioactive compounds in plants extracts. Key analytical techniques such as thin layer chromatography (TLC), column chromatography, flash chromatography, high performance thin layer chromatography (HPTLC), and high performance liquid chromatography (HPLC) have relevant advancements in separations. Pure compounds of the colourant extract are further analysed using techniques such as Ultraviolet/Visible (UV-Vis) spectrometry, Fourier transformed–infrared (FT-IR), NMR and mass spectrometry where in each method the colourant is prepared independently. UV-VIS and FT-IR spectroscopies are used as qualitative tools to characterise the sample

as per the absorption spectra of the colourant and; and identify their molecular structures (Espinosa, Reyes, Hermosín, & Azamar-Barrios, 2012) and these shall be applicable in this study of *B. vulgaris* peels and pomace extracts.

2.9.1 UV-Vis Spectrophotometry

In UV-Vis spectroscopy, the hue and absorbance of the dye is determined by using UV-Vis spectral scan of aqueous or non-aqueous extract of purified natural dye. The UV and visible zone range from 190 to 700 nm or higher. Peaks and troughs in different wavelength indicate the presence of dye within this range. Those in visible zone indicate the main colour due to absorption, while the UV zone (with or without peaks) shows the property of the dye under UV light (Razak, Tumin, & Tajuddin, 2011). This can be correlated with its fastness behavior. In the study of historical and archaeological textiles, an insight on degradation products of wool and silk yarns, UV-VIS method proved to be realiable and cheap than MS method (Degano, Biesaga, & Colombini, 2011).

2.9.2 High Performance Liquid Chromatography (HPLC)

In HPLC the compounds or active ingredients are separated on the basis of their interaction with the solid particles of a tightly packed column and the solvent of the mobile phase. This technique is useful for compounds that cannot be vaporized or that decompose under high temperatures. HPLC method provides both qualitative and quantitative measurements simultaneously. The HPLC technique coupled with a UV photodiode array detector or mass spectrometer provides more structural information on the compounds. In HPLC analysis of indigo dye in dyeing process, Song, Jing, & Rui (2012) highlighted the structural changes of the indigo component and attributed the

decrease and increase in color strength with variation of dyeing time. Furthermore, HPLC method was developed and used in the analysis of natural red dyes (cochineal) in textiles of historical importance (Serrano, Sousa, Hallett, Lopes, & Oliveira, 2011).

2.9.3 High Performance Thin Layer Chromatography (HPTLC)

This method is slightly advanced than HPLC. It is a planer chromatography whereby separation of the sample components is achieved on high performance layers with detection and data acquisition using an advanced work station. This is a robust, rapid and an efficient tool in quantitative analysis of compounds. It is based on TLC and comprises of several enhancements with the objective of increasing the resolution of the compounds to be separated for quantitative analysis (Chernetsova & Morlock, 2012). HPTLC technique utilizes high quality TLC plates with finer particle sizes in the stationary phase for better resolution. The plates can be constructed as repeated mode to allow improved separation of compounds by using a multiple development device. There are better improvised hyphenated techniques such as HPTLC-Direct Analysis in Real-Time-Of-Flight Mass Spectrometry (DARTTOF-MS) has ever been used, for instance in rapid analysis of oxidative degradation of an azo dye. The oxidation reaction products were clearly identified by this method (Djelal, Corn´ee, Tardivel, Lavastre, & Amrane, 2013). HPTLC-DART-MS technique is a new technique useful in diverse application, such as ascertaining food and dye quality, safety control (Chernetsova & Morlock, 2012), biological fluids, tissues, drugs and narcotics analysis, and rapid analysis of crude products (Chernetsova & Morlock, 2012; Zeng *et al.*, 2012; Shena *et al.*, 2012).

2.9.4 Thin Layer Chromatography (TLC)

Advantage of TLC is its versatility, speedy and sensitivity. TLC is an adsorption chromatography technique in which samples are separated based on the interaction between a thin layers of adsorbent attached on the plate, and mostly used in separation of low molecular weight compounds. This method is mostly applicable in rapid analysis of plant extracts with minimum sample clean-up requirement. It also provides qualitative and semi quantitative information of the separated compounds and the quantification of chemical constituents.

Different adsorbents such as aluminium, cellulose powder, silica gel and starch can be utilized in separation of various molecular compounds for instance amino acids, alkaloids, phenols, steroids, vitamins etc. There have been studies on use of TLC such as in separation and identification of some synthetic food colorants from foods through thin layer chromatography – UV/VIS spectrometry (Măruţoiu *et al.,* 2011). Development and validation of a TLC method for the analysis of synthetic food-stuff dyes is another case study on TLC utilization as a fast and flexible method (Vlajković *et al.,* 2013).

2.9.5 Column Chromatography (CC)

Column chromatography involves use of a column with ion exchange, molecular sieves, and adsorption phenomenon. The column is prepared using silica for column chromatography. The flushing in conventional chromatography greatly dilutes the material, and the fractions usually require another step for concentration. A newer method known as displacement chromatography can elute with the compounds that has great affinity for the adsorbent. Fractions of eluted materials can be of higher concentration than the original solution applied to the column. The fraction is dissolved

in smallest possible volume of solvent and mixed with 2 grams of silica. The mixture is dried to obtain free flowing powder and added to column. Then the column is eluted with solvent of various proportions. The eluent is collected in properly cleaned test tube. In isolation for structural elucidation of flavonoids from *Polyathia longifolia* and evaluation of antibacterial, antioxidant and anticancer potential, column chromatography was used extensively to select the best solvent system (Sampath & Vasanthi, 2013). Similary, the method was applied in comparative evaluation of antimicrobials for textile applications (Windler, Height, & Nowack, 2013).

2.10 Detection Methods

FT-IR is a known and valuable tool for the identification and characterization of compounds or functional groups (chemical bonds) present in an unknown plant extract. The method is commonly applied in screening techniques such as in food fraud, especially FTIR based on mid-infrared (MIR) vibrational spectroscopy (Georgouli, Del Rincon, & Koidis, 2017); Moore, Spink, & Lipp, 2012). Its offers a rapid and reliable detection method since pure compounds usually are so unique that they are normally regarded as molecular "fingerprint". A typical FTIR spectra show several absorbance peaks due to fundamental transitions. They are distributed discriminately in four regions: the -OH stretching region (4000–2500 cm^{-1}), the triple-bond region (2500–2000cm^{-1}), the double-bond region (2000–1500 cm^{-1}) and the fingerprint region (1500–600 cm^{-1}) (Szymanska-Chargot, Chylinska, Kruk, & Zdunek, 2015). The spectrum of an unknown plant compound can be identified by comparing with the known compounds in the infrared spectral library. Samples for FTIR can be prepared in a number of ways; liquid samples prepared by having one drop of sample between two plates of sodium chloride

that forms a thin film between the plates. Solid samples are prepared by milling with potassium bromide (KBr) and then compressed into a thin pellet that can be analyzed. The KBr inorganic salt does not generate any vibrations in the MIR region (from 4000 to 400 cm^{-1}). It is commonly used for determination of most organic molecules (Nyquist & Kagel, 2012). The region in IR spectrum above 1200cm^{-1} shows spectral bands or peaks due to the vibrations of individual bonds or functional groups under examination. The region below 1200 cm^{-1} indicates bands due to the vibrations of the whole molecule and because of its complexity is known as the 'Fingerprint region'. Intensities of the various bands are recorded subjectively on a simple scale as being either strong (S), medium (M) or weak (W) (Nyquist & Kagel, 2012).

Mass spectrometry (MS) is also a powerful analytical technique for the identification of unknown compounds, quantification of known compounds and to elucidate the structure and chemical properties of molecules. The MS spectrum can provide the molecular weight of a sample. The value of the technique is that it requires only small quantities or microgram amounts of sample. Gas chromatography equipment can be directly coupled with rapid scan mass spectrometer (MS) of various types. High-resolution analysis can be performed by coupling or hyphenating the equipment. For example, Liquid Chromatography-Mass Spectroscopy (LC-MS) offers accurate determination of molecular weight and isotopes patterns of proteins or peptides. To fit modern high accuracy, sensitivity, and selectivity needs, the rapidly advancing MS instrumentations are emerging at the forefront of analyzing analytes with limited alternative biological assays (Xu *et al*., 2019; Nowak *et al.*, 2017; Ambrogelly *et al*., 2018), biotransformation, and high-throughput screening (HTS). In order to achieve relatively better throughput

results, matrix-assisted laser desorption/ionization (MALDI) is compatible with speeds >100,000 samples per day. Through incorporating self-assembled monolayers desorption/ionization (SAMDI) time-of-flight (TOF) technology (VanderPorten, Scholle, Sherrill, Tran, & Liu, 2017), it is possible to infer small molecule noncovalent identification, but larger molecular weight compounds (e.g. proteins) may have limited mass resolution and quantitative challenges.

Nuclear Magnetic Resonance Spectroscopy (NMR) provides physical, chemical and biological properties of matter. An example is carbon 13 NMR (^{13}C NMR) that is used to identify the types of carbon present in a compound. ^{1}H- NMR or proton NMR is a method used to find out the types, inter-connection and magnetic moments of hydrogen atoms present in a compound, consequently determining the structure of the compound. In most compounds, hydrogen atoms are attached to different groups (as -CH_2-, -CH-, -CHO, -NH_2, -CHOH-, etc) and the proton NMR spectrum provides a record of the number of hydrogen atoms in these different situations. However, it cannot give any direct information on the nature of the carbon skeleton of the molecule; this can only be obtained by carbon 13 NMR spectroscopy. ^{13}C-NMR spectroscopy is complementary to proton NMR. The combination of the two techniques provides a very powerful means of structural elucidation for new terpenoids, alkaloids or flavonoids. It is useful in the analysis of glycosides, in indicating the linkage between sugar moieties and their configurations. Both proton (^{1}H) and ^{13}C-NMR measurements have been successfully applied to structural and other analyses of proteins and other macromolecules.

During the identification of polish cochineal (*Porphyrophora Polonica* L.) in historical textiles, high-performance liquid chromatography coupled with spectrophotometric and tandem as spectrometric detection was applied (Lech & Jarosz, 2016).

2.11 Qualitative Techniques

2.11.1 Detection of Alkaloids

B. vulgaris plant contains mainly betanins that are alkaloids and anthocyanins. For alkaloids determination, Mayer's test is performed where two drops of Mayer's reagent are added along the sides of test tube containing a few amount of plant extract. The presence of alkaloids is indicated by a white creamy precipitate (Banu & Cathrine, 2015). Wagner's test can also be conducted to ascertain the presence of alkaloids (Banu & Cathrine, 2015). A few drops of Wagner's reagent are added to a few amount of plant extract and a reddish brown precipitate depicts the presence of alkaloids. While in Dragendroff's test, few drops of Dragendroff's reagent is added into the extract and appearance of red precipitate indicates the presence of alkaloids in the sample (Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011). Another alternative can be Hager's test. A small amount of Hager's reagent is added to the plant extract and the formation of yellow precipitate indicates the presence of alkaloids (Tiwari *et al.,* 2011).

2.11.2 Detection of Carbohydrates and Glycosides

In this case, 100 mg of extract is dissolved in 5 mL of distilled water and filtered. Then Molish's test is done. Two drops of alcoholic solution of α–naphthol are added to 2 mL of filtrate and 1 mL of concentrated sulpuric acid is added slowly to trickle along the sides of test tube. A violet ring indicates the presence of carbohydrates. Fehling's test is another option. An equal volume of Fehling solution A and B are added to an equal

volume of filtrate and it should boil in a water bath. The formation of red precipitate indicates the presence of sugar, which are carbohydrates.

Barfoed's test by use of equal volumes of filtrate and Barfoed's reagent. They are mixed and heated in a water bath whereby a red precipitate indicates the presence of sugar. Benedict's test also indicates the presence of carbohydrates. A mixture of plant extract and the Benedict reagent is heated on water bath for 2 minutes and a characteristic coloured precipitate indicates the presence of sugar.

For the detection of glycosides, the plant extract is hydrolyzed with concentrated hydrochloric acid and the filtrate should be subjected to the following tests; Borntrager's test where 2 mL of plant filtrate is mixed with 3 mL of chloroform and 10% ammonia is added to the mixture. A pink color solution indicates the presence of glycosides. In Legal's test, plant extract is dissolved in pyridine and sodium nitroprusside is added. Then the solution is made alkaline by adding 10% sodium hydroxide and pink color solution proves the presence of glycoside.

2.11.3 Detection of Saponins

The plant extract around 50 mg is diluted with distilled water up to 20 mL and then shaken or sonicated for 15 minutes in a graduated cylinder. The formation of a thick foam (2 cm) is an indicator for the presence of saponins (Banu & Cathrine, 2015).

2.11.4 Detection of Proteins and Amino Acids

While detecting the presence of proteins and amino acids in a plant extract, the extract is dissolved in 10 mL of distilled water and the filtrate is used for the following tests; Millon's test is undertaken whereby a few drops of Millon's reagent is added to 2 mL of

filtrate. White precipitate indicates the presence of proteins. In a Biuret test one drop of 2% copper (II) sulphate solution is added to 2 mL of filtrate of the extract, and then 1 mL of 95% ethanol is added. Excess of potassium hydroxide pellets is finally added. Pink color in ethanolic layer is a proof that proteins are present. Ninhydrin test is also performed by adding two drops of ninhydrin solution to 2 mL of the filtrate and purple color proves the presence of amino acids. As per Xanthoproteic test, the plant extract is treated with few drops of concentrated nitric acid. The formation of yellow color is a clear indicates of the presence of proteins (Tiwari *et al.*, 2011).

2.11.5 Detection of Flavonoids

Alkaline reagent test is conducted to ascertain the presence of flavonoids. A small quantity of the extract is treated with few drops of sodium hydroxide. The intense yellow colour solution becomes colorless on addition of dilute acid becoming an evidence that flavonoids are present (Saxena M., Saxena J., Nema, Singh, & Gupta, 2013). In Lead acetate test, the plant extract is treated with few drops of lead acetate solution and the formation of yellow color solution indicates the presence of flavonoids (Tiwari *et al.*, 2011). Magnesium and hydrochloric acid reduction test also proves flavonoid presence. A small amount of extract (50 mg) is dissolved in 5 mL of ethanol and few fragments of magnesium ribbon together aith a few drops of concentrated hydrochloric acid is added. The development of any pink to crimson color indicates the presence of flavanol glycosides.

2.11.6 Detection of Phytosterols

Libermann-Burchard’s test is used to determine the presence of phytosterols. A small amount of the extract (about 50 mg) is dissolved in 2 mL of acetic anhydride. A few

drops of concentrated sulphuric acid is added and the colour change is monitored. An array of color change shows the presence of phytosterols. Salkowski's test when done to show the presence of phytosterols, the extract is treated with chloroform, filtered and a few drops of acetic anhydride is added. The solution is then boiled and cooled. Formation of brown ring at the junction indicates the presence of phytosterols (Tiwari *et al.,* 2011).

2.11.7 Detection of Phenols and Tannins

In the detection of phenols, the plant extract is treated with few drops of ferric chloride solution and the formation of bluish black color proves the presence of phenols (Tiwari *et al.,* 2011). For tannins, a few drops of 1% gelatin solution containing sodium chloride is added to the plant extract. The formation of white precipitate indicates the presence of tannins (Tiwari *et al.,* 2011).

2.11.8 Detection of Diterpenes

Copper acetate test is a prominent test for these phytochemicals. The plant extract is dissolved in water and 3-4 drops of copper acetate solution is added. Formation of emerald green color indicates the presence of diterpenes (Tiwari *et al.,* 2011).

2.12 Application of Natural Dye and Mordants on Cellulosic Fabrics

Cotton is a natural fiber that obtained from the seedpod of the cotton plant. The fiber is hollow in the center and, under the microscope, it resembles a twisted ribbon (Kljun *et al.,* 2014). Cotton and cellulosic fibers comprise over 40% of textile consumption all over the world. Colour can be imparted to cellulosic fabrics using different classes or types of dye, with natural dyes finding its use with greater acceptance. Cellulose is tasteless and odorless. It is hydrophilic hence insoluble in water and most organic solvents. It is biodegradable

as it consists of crystalline and amorphous regions that can be broken down chemically into its constituent glucose units (Abidi, Cabrales, & Haigler, 2014). This can be achieved by subjecting cellulose to concentrated inorganic acids at relatively high temperatures to produce nano crystalline cellulose, a novel material with many desirable properties (Abidi *et al.*, 2014).

Cellulose from cotton and other plant fibers as well as bacterial cellulose has chain lengths ranging from 800 to 10,000 units. The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ where n is the degree of polymerization and represents the number of glucose units (Figure 2.10). Its molecular weight is 342.297 g/mol (Abidi *et al.*, 2014).

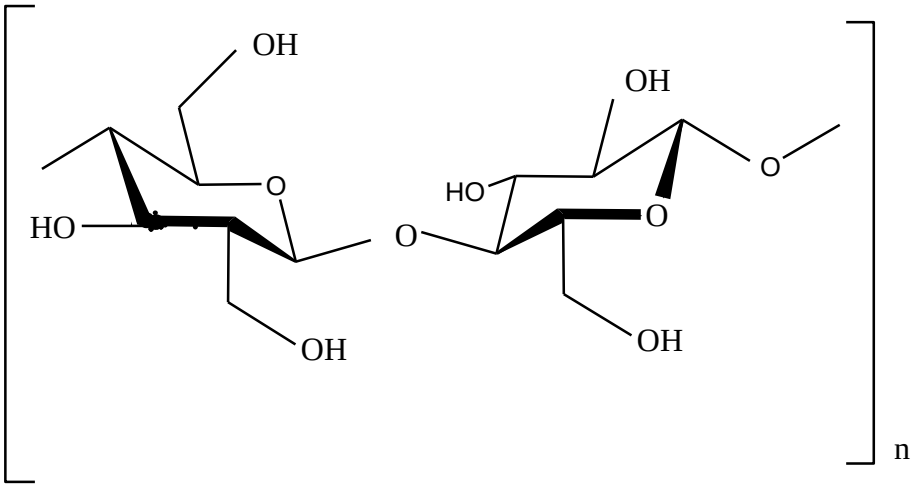


Figure 2.8. Chemical structure of cellulose.

Many researchers have reported on the available methods of dyeing fibers such as cellulosic, protein and synthetic with different synthetic and natural dyes. For example, Prabhavathi, Devi, & Anitha (2014) improved the colourfastness of certain natural dyes by optimizing the dyeing conditions of cotton fabrics. His study established that with increase in mordant concentration the shade became darker (Prabhavathi *et al*., 2014). A

study by Kulkarni *et al.* (2011) involved dyeing of cotton with natural dye extracted from Pomegranate *Punica granatum* peel.

Kumaresan, Palanisamy, & Kumar (2012) wetted out silk samples, dipped them in a natural dye bath, and carried out dyeing for one hour at 60^0C (Kumaresan *et al.*, 2012). Researchers have performed dyeing of natural fibres under different parameters; dyeing temperature, time or duration of dyeing, concentration of salt and mordant, pH, type of mordant, dyeing procedures, type of fabric and fabric processing techniques. Monitoring of dyeing time, temperature, pH and concentration of mordant have been proven as the most essential factors (Gordon, 2015).

2.12.1 Optimization of Dyeing Process Conditions

Researchers have developed approaches to study dyeing parameters or conditions for optimum results. Such effects include dyeing temperature, time, pH, conductivity, sampling rate and concentration of salt and mordant (natural or synthetic). The nature of the dye dictates the technique of dyeing. Ali & El-Mohamedy, 2011 established that the colour strength of wool fabrics dyed with red prickly pear dye increased with increase in temperature (Ali & El-Mohamedy, 2011).

Razak *et al.* (2011) used Pressurized Hot Water Extraction Method (PHWE) to study the effect of varying temperatures (50, 75, 100 and 125 $^{\circ}$C) on the colour of natural dyes from Possur plant. At different temperatures, different colour shades were obtained. In addition, temperature also affected the extraction efficiency and solubility (Razak *et al.*, 2011).

Increase in temperature may result into increased destruction of pigments such as betalains and anthocyanins. Therefore, to obtain substantial results, the dyeing temperature of natural dyes with *B. vulgaris* or anthocyanins should range from 60- 90 °C. This is so because high temperatures accelerate degradation of the betalains or anthocyanins (Lioe, Adawiyah, & Anggraeni, 2012).

Response Surface methodology (RSM) is a statistical design tool aimed at process optimisation, design of experiments (DOE) and analysis (Hamanthraj, Desai, & Bisht, 2014). Conventional methods are disadvantageously time consuming and incapable of detecting the true optimum condition of a dye. This is because of the absence of interactions among factors and defining the effect of the independent variables alone, or in combination with the processes (Kaur, Kumar, Garg, & Navneet, 2014). RSM design include central composite design (CCD) and Box-Behnken design (BBD) of experiments that have been applied for optimisation of extraction and dyeing parameters.

The RSM design adopts a DOE and depends on the number of design variables or factors. CCD gives a total number of ten factors ranging from two to ten but for BBD it excludes 2 and 8. CCD codes the factors into five levels as -1.414, -1, 0, +1 and +1.414 resulting into many possible experimental runs. BBD codes the factors into three levels as -1, 0 and +1 creating designs with desirable statistical properties (Yim *et al.*, 2013).

2.12.2 Mordanting of Natural Dyes

Mordants are substances used to adhere dyes on fabrics. Mordant possesses affinity for both fibre and dye. They act by reacting with the dye and together forming a coordination complex then attaches to the fabric. The word mordant comes from the Latin *mordere*,

meaning to bite. Mordants fix and modify colour onto fabrics as well as enhancing the colourfastness of dyed fabrics (Ghouila *et al.,* 2012; Konar & Samantha, 2011; Kulkarni *et al*., 2011). Some mordants can be used for dyeing fabrics alongside acting as a fixating agent. The metal-dye complexation increases dye uptake, hence dye concentration rises on the fabric surface. Dye–mordant complexation is generally accompanied with colour changes due to the formation of charge transfer complexes (Pyrzynska & Pekal, 2011).

Natural dyes need mordants (metal ion) for absorption and fixation of dye. Mordant and day form an insoluble composition precipitate on the surface of the fibres and this give the fabric a range of bright colours (Uddin, 2015).

Metal salts are commonly used as mordants. The selection of mordant and techniques of mordanting are influenced by the type of substrate, mordant affinity to the substrate and the type of dye in use. A number of research work has been documented for the natural dyeing of various textile materials while applying certain mordanting techniques (Basak *et al.,* 2012; Kaur *et al.*, 2014; Lakshmi, 2015; Lokesh & Kumara, 2013; Prabhavathi *et al.*, 2014; Saha & Sinha, 2012; Saxena & Raja, 2014; Wangatia *et al.*, 2015; Yim *et al.,* 2013).

However, mordanting with salts such as chrome, copper and tin have been proven to cause health hazards as they produce toxic waste which requires special disposal hence not recommended for use. On the other hand, metallic mordants such as iron as well as natural mordants like alum and tannins are safer to humans and environment. Both metallic and natural mordants are capable of producing innumerable colours when used in conjunction with natural dyes. They are biodegradable and naturally occurs in the

environment in abundance (Konar & Samantha, 2011; Saxena & Raja, 2014). Plant polyphenols such as tannins or tannic acid function as natural mordants (Ghaheh, Nateri, Mortazavi, Abedi, & Mokhtari, 2012). Other natural mordant sources include myrobolan obtained from *Terminalia chebula* fruits, mango bark from *mangifera indica* (Wangatia *et al.*, 2015). Lemon juice and vinegar (Prabhu & Bhute, 2012). Very little attention has been paid on improving colourfastness with natural fixing agents. However, some studies have been done such as Prabhavathi *et al.* (2014) who used five fixing agents namely vinegar, limejuice, alum and myrobolan to mordant fabrics using eucalyptus bark dye,whereby vinegar exhibited very good fastness results among the agents (Prabhavathi *et al.*, 2014).

The choice of mordant depends upon the fabric to be dyed. In order to improve the washing fastness of natural dyes, treating them with eco-friendly mordants such as alum, tannic acid or sumac and alkali mordants(e.g. soda ash) is essential (Saxena & Raja, 2014).

Oil mordant are in existence and it form a complex with alum when it is used as the main mordant. Oil is insoluble in water and has poor affinity for cotton hence will not easily be washed out from the treated fabric surface. Oil mordants contains fatty acids such as oleic, palmitic, stearic, ricinoleic and glycerides. During mordanting, the -COOH group of the fatty acids reacts with metal salt leading to the formation a COO-M, where M denotes the metal. Among the oil available, sulphonated oils are well known due to their better metal binding capacity than the natural oils and they have found use in dyeing of madder to obtain Turkey red colour (Kumaresan *et al*., 2011; Saxena & Raja, 2014).

A study on plant species that grow in Malaysia by Soheil, Abu Bakar, & Norasikin (2017) for the extraction of betalain dye from it is crust showed that betalain dye was soluble in different solvents (i.e., methanol, hexane, ethanol, and acetonitrile). Ultraviolet-visible spectrophotometry (UV-Vis) and Fourier transform infrared spectroscopy (FTIR) were used to study the characteristics of the extraction in different solvents, pH, and temperature. The results identified that hexane was the best solvents to use for dye extraction.

Jain (2013) extracted colourant from jamun leaves, bark, bark peel and fruit and dyeing was carried out on cotton pre-mordanted with natural mordants; amar chal, arjun chal and babul barkat following conditions; dye extraction time of 45min., dye material concentration of 20mL/150mL water, mordanting time of 60 min., mordanting temperature of 60 °C, dyeing time of 60 min. and dyeing temperature of 60-75 ºC. The jamun leaves mordanted with babul bark demonstrated better shades than the others.

Mordants are agents applied onto substrates before, during, or after the coloration step (termed pre-, meta/simultaneous-, and post-mordanting respectively). In Pre-mordanting, the textile fabric is first immersed into the mordant solution and then dyed with the extracts. The dyed material is then washed with a non-ionic detergent. The major advantage of this method is that the dye baths in use can be re-used many times after replenishing the mordants. This makes the process economical and reduces pollution content hence sufficient for large- scale production (Saxena & Raja, 2014). The better results obtained in case of pre-mordanting with stannous chloride and ferrous sulphates are attributed to the empty d-orbitals of ferrous and stannic ions.

Simultaneous mordanting entails immersing the fabric in a dyebath solution containing both mordant and the dye extract. Both mordanting and dyeing processes take place concurrently (Saxena & Raja, 2014). Dyeing chemical auxiliaries can be added during the process of dyeing. After dyeing, the textile material is then washed with non-ionic detergent.

In post-mordanting method, the dyeing process is carried out on the scoured and bleached textiles in the dye-bath without mordant. The dyed fabric is then treated with another bath called saturator containing the mordanting solution. Treatment condition may vary depending on the type of fibre, dye and mordant system being used. After dyeing, the textile material is washed properly with non-ionic detergent. When using this mordanting method, the colours are usually different and often less strong often black to grey shades (Saxena & Raja, 2014).

2.12.3 Mechanism of Dyeing

The chemistry of bonding between dyes and fabric or fibers is complex involving direct bonding, hydrogen, H-bonds and hydrophobic interactions. To this effect mordants increase binding of dye to fabric by forming a chemical bridge from dye to fabric (Ghouila *et al.* 2012; Konar & Samantha, 2011; Kulkarni *et al*., 2011) as seen in Figure 2.9.

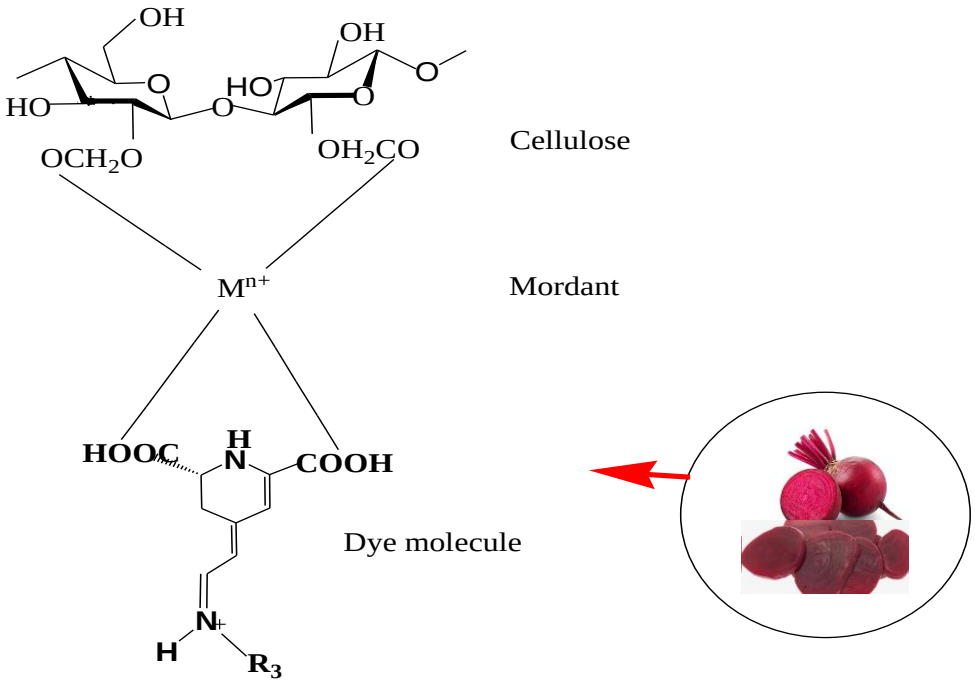


Figure 2.9. Chemical bridge between cellulose, mordant and dye.

The mordant should have affinity for both fabric fiber and the dye. However, those dyes that do not have any affinity to a fiber can be applied by using mordants to fix the dye. Therefore, this improves the dye reception ability of any dye at the same time increasing its fastness properties.

CHAPTER THREE

METHODOLOGY

3.1 Chemicals and Reagents

Methanol, formic acid, copper (II) sulphate, potassium dichromate, tannic acid, alum, hydrochloric acid and acetic acid were acquired from Loba Chemie; Iron (II) sulphate (Blulux); Sodium hydroxide, Gulbar salt or sodium sulphate, sodium carbonate (Narcolab); Reactive Orange HER-C.I. 84 (Roop); Penetrol HPLF-E wetting agent (Pacific Texchem); Betanin standard (Betanidin 5-β-D-glucopyranoside standard), CAS number 53158-73-9, lot number CFS201901 , percent purity >= 95% (Wuhan ChemFaces Biochemical Co., Ltd, China); Sodium formate and 2, 2- difluoroethoxy phosphazene (Fisher Scientific, USA). All the acquired chemicals and reagents were of analytical grade and were used without any further purification.

3.2 Equipment

Soxhlet apparatus and Whatman cellulose extraction thimbles, Whatman filter paper, qualitative, Grade 1, 11 μm (Sigma Aldrich, Germany); Standard Portable PX Meter (0.1 pH Resolution); Rotary vacuum evaporator (Hahnvapor Rotary Evaporator HS-2005S, Korea); UV-Vis spectrophotometer (Beckman Coulter DU 720 UV/Visible Spectrophotometer, USA); TLC plates-60RP-18 (Sigma Aldrich); Standard swatch master, Spray master (with AATCC certified standard spray Test Rating Chart), dye water bath, padding mangle, spectra vision (5L) color matching booth, launder-o-meter wash master, crockmaster, light fastness MBTL sun master (all from Paramount Instruments Pvt Ltd, India); Perspirometer (SDL Atlas,USA) and Reference Bench-top

Spectrophotometer (SP60 X-Rite, USA); FTIR (Bruker Alpha, USA, equipped with Platinum ATR beam splitter, with monolithic diamond crystal); Nylon syringe filter, 0.45 μm, 25 mm (Millipore, USA); HPLC (LC-10AT VP Shimadzu) equipped with SPD-20A UV detector and column (Supelcosil LC-18,5 UM, Dimension: 15 cm by 4mm, USA); Thermo Scientific Triple Quadrupole Mass Spectrometer (TSQ) Altis fitted with an OptaMax NG and coupled to a Vanquish Horizon UHPLC with Thermo Scientific Hypersil GOLD aQ™ column (50 x 2.1 mm, 1.9 um).

3.3 Sample Collection

The plant sample (*B. vulgaris* plant peels and pomace) were collected in Eldoret town (latitude 0° 30' 51.4" N, longitude 35° 16' 11.2" E); from restaurants and fruit juice vendors. Care was taken to collect fresh and only pure *B. vulgaris* peels and pomace to avoid mixing with other vegetable matter. They were then placed in sample box (Polyethylene Chest Cooler) lined with polypropylene interior for optimum temperature and humidiy. The fresh peels and pomace were finally placed in perforated polybags and stored at -4 $^{\circ}$C in Moi University chemistry laboratory. Use of purposeful sampling method was largely employed in this study. Stratified random sampling method was applied in collection of materials scoured and bleached 100 % cotton fabric at Rivatex East Africa Ltd, Processing department. There are various 100% cotton fabrics at the department, with diverse picks/cm, ends/cm, warp and weft counts as well as grams per square metre (GSM). Purposeful sampling was eventually used in the collection of cotton fabrics, in particular an article that is lighter for easy dye absorption. The fabric in consideration had 25.5 ends/cm, 20 picks/cm, NM 46 count (warp and weft) and 98.9 GSM.

3.4 Sample Preparation

The fresh peels and pomace were further sliced into small pieces and dried in an oven. Since heat destroys beetroot pigments, the sliced peels and pomace were spread over a layer of wax paper and covered with the same wax paper. The samples were then placed in an oven and dried at 40 °C (104 °F), with the oven-fan on and the door slightly open to allow moist air to escape. This process was undertaken for 5 hours to be completely dry and crunchy. Afterwards the dried plant materials were crumbled using pestle and mortar into very fine powder (Appendix 1) and stored in airtight containers in cool and dry environment.

3.5 Scouring and Mercerizing of Cotton Fabrics

Before dyeing, grey cotton fabrics were modified through desizing, scouring, bleaching and mercerization (Hong, 2018; Bhuiyan *et al.* 2017). Desizing is a procedure in textile industry used to remove size (such as starch, beef tallow etc) especially in cotton fabrics by use of chemicals such as sodium hydroxide (NaOH), sodium silicates and hydrogen peroxide or by use of enzymes such as amylase. Scouring is the process of removing impurities from fabric surface that may prevent effective dyeing, for example fats, oils, waxes, stubborn dirt and vegetable matter by subjecting the fabric to hot bath of NaOH and soda ash. Scouring on bulk fabric was done at Rivatex East Africa Ltd in a hot solution of sodium hydroxide (5g/L) and sodium carbonate (3 g/L) in presence of wetting agent (penetrol HPLF-E), at temperature of 100 °C for 30 minutes. Soda ash helps to adjust pH to 10.5 required in scouring. Mercerizing is a special treatment of fabrics with NaOH solution in order to alter the chemical structure of cotton fibre with the aim of making it lustrous, shiny and to allow effective absorption of dyes. NaOH treatment

renders the cotton surface rough and increases fiber strength for improved mechanical interlocking as well as increasing the amount of cellulose exposed on the fiber surface. Mercerization procedure was performed after scouring by padding the bulk fabric in a solution of sodium hydroxide (NaOH) at standard mercerization concentration of 30 (i.e. Twaddle scale of 52 ºTw or 30 ºBé - approximately 270-330 g/L) with a dwell time of 30-60 seconds. The treatment was done at room temperature and fabric under specified tension (Hong, 2018). The mercerized fabrics were then washed with non-ionic detergent at 50 ºC for 25 minutes and dried at room temperature. From a scoured and mercerized 100% plain woven cotton fabric, twenty (20) plain pieces of dimensions 10 x 15 cm with average weight of 2.0 g were obtained to be used in this experimental study.

3.6 Experimental Study Design

A conceptual summary of the methodology used is given in Figure 3.1.

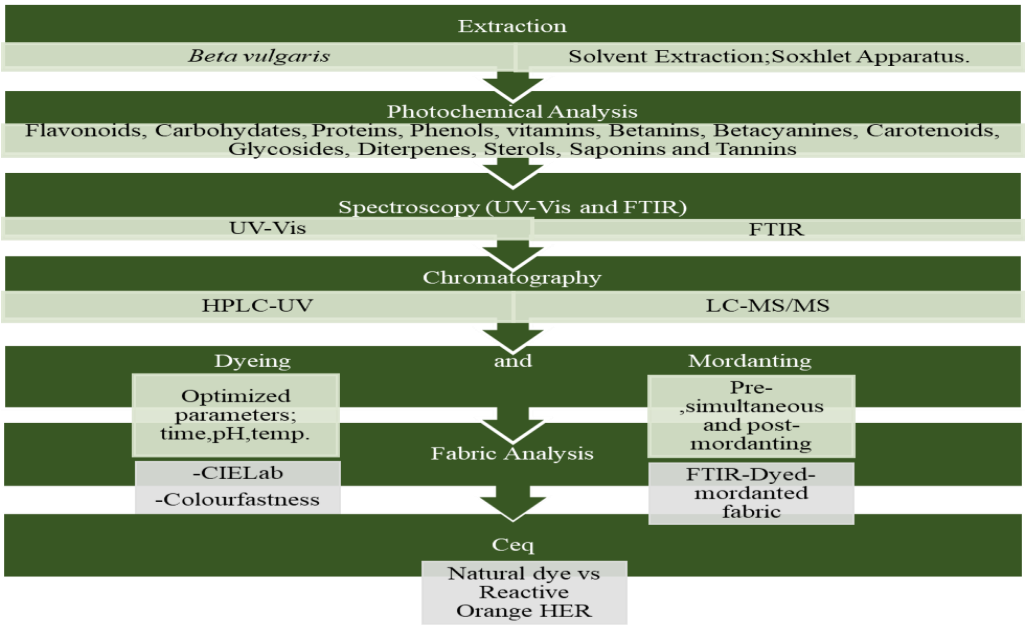


Figure 3.1. Conceptual summary of the methodology.

3.7 Statistical Analysis

Minitab 17 statistical software, Central Composite Design (CCD) of Response Surface Methodology (RSM) was used to design 14 and 20 experiments for extraction and dyeing respectively. Optimization is critical to determine the best design mechanism for pathways and systems (Rather *et al.* 2016). Parametric inferential and predictive regression statistical methods by way of analysis of variance (ANOVA) and Microsoft Excel software were used for regression analysis respectively for the levels of input variables that influence and optimize the responses of extraction and dyeing (Hamanthraj *et al.*, 2014; Nasirizadeh, Dehghanizadeh, Yazdanshenas, Moghadam, & Karimi, 2012). Predictive correlation method (Pearson's correlation) was applied in relating the effects of the variables. The results were expressed as mean ± standard deviation (SD) (n=3). The statistical probability (p-value) less than 0.05 indicated a statistical difference between groups. The percentage yield, betalain quantities, mordants effects and colour equivalence estimations were done in triplicates and results presented on respective charts and bar graphs for descriptive statistics.

3.8 Optimization of Extraction of *B. vulgaris* Peels and Pomace Natural Dye

Optimization of the conditions such as combination of plant material and solvent (material to liquor ratio i.e. MLR) and extraction time was standardized for maximum extraction of natural dye from *B. vulgaris* plant peels and pomace. While adopting RSM and CCD, the extraction procedures were performed following three factor levels and subsequent experimental runs using time and M: L variables (Hamanthraj *et al.,* 2014; Nasirizadeh *et al.,* 2012). Table 3.1 shows the actual uncoded variables where A and B signify dyeing time (hours) and M: L ratio (o.w.f) respectively. The experimental runs

alongside coded factors, the amounts of solvent used and extraction time are found in Table 3.2. The dried *B. vulgaris* peels and pomace material (20 grams) was used consistently in all the experiments. The factor variables are evaluated independently at three levels denoted by (+1), (0) and (-1), respectively i.e. high, mid-point and low-point respectively.

Each dye extraction experiment was done in Soxhlet apparatus having round bottom 500 mL two-neck Schlenk flask with glass stopcock (Appendix 2). Finely sieved *B. vulgaris* peels and pomace powder (20 g) were taken and carefully filled into Whatman cellulose extraction thimble. The thimble was then inserted into the Soxhlet apparatus with care so that it slides gently and no drops of powder spilling over. Spillage may cause clogging of the apparatus. The apparatus was then assembled with glass joints slightly loose over a heating plate. The Schlenk flask was then filled to one third with 200 mL methanol and few drops of formic acid added to prevent colour degradation of the extracts. The stability of betanin depends directly on its pH, the optimum pH being between 4 and 5 (Antigo, Rita de Cássia, & Grasiele, 2017). After the set up was complete i.e. all siphons to and from the condenser and clamping safely, the hot plate was put on. Extraction was carried the boiling point of methanol (68 °C) solvent guided by the experimental runs. The dye extracts obtained from each of the experiments were cooled, filtered using Whatman No. 1 filter paper (11 μm). Concentration followed by Vacuum Rotary Evaporator to obtain crude extracts.

Table 3.1. The factor levels and variables.

Variables		Coded factor levels				
Symbol	Name	-α	-1	0	+1	+α
A	Time	5.17	6	8	10	10.8
B	M:L	1.89	5	10	20	23.1

Table 3.2. Randomized Experimental runs.

Run	A	B	Amount of solvent (ml)	Extraction time (hrs)
1	0.00000	0.00000	200	7.5
2	-1.00000	1.00000	400	5.0
3	1.00000	-1.00000	100	10.0
4	1.00000	1.00000	400	10.0
5	-1.00000	-1.00000	100	5.0
6	0.00000	-1.41421	80	7.5
7	-1.41421	0.00000	200	4.0
8	1.41421	0.00000	200	11.0

3.9 Extraction Yield Calculation

The concentrated dye samples were transferred to weighed 250 mL beakers and dried in an oven at 40 ^{o}C to evaporate the solvent. After this, they are weighed to determine the weight of the extract from which extraction yields were calculated as indicated in Equation (3.1) according to Umaru I., Badruddin, & Umaru, H. (2019);

$$Yield\ (\%) = \frac{(W1*100)}{W2} \qquad Equation\ (3.1)$$

Where, W1= weight of concentrated extract residue, W2= weight of the plant powder.

3.10 Statistical Modelling of Extraction process

A second order polynomial equation was used to express the betalain content in each extraction as a function of independent variables, which affects extraction.

$$Response,\ y = b0 + (b_1 F_1) + (b_2 F_2) + (b_3 F_3) + (b_4 F_1 F_1) + (b_5 F_2 F_2) + (b_6 F_3 F_3) + (b_7 F_1 F_2) + (b_8 F_1 F_3) + (b_9 F_2 F_3) \qquad Equation\ (3.2)$$

In the Equation (3.2), *y* represents the betalain content, (unit: mg/g) while *b* and *F* are the coefficients and factor variables respectively.

3.11 Preliminary Qualitative Phytochemical Analysis

The *B. vulgaris* peels and pomace extracts were subjected to phytochemicals screening for determination of plant secondary metabolites following the standard procedures adapted by Vulić *et al.,* 2012. The major phytochemical components that were considered for screening were carbohydrates, vitamins, proteins, betanins, betacyanines, carotenoids, flavonoids, glycosides, diterpenes, sterols, phenols, saponins and tannins.

3.12 Thin Layer and Column Chromatography

Several solvents (from polar to non-polar) were used to establish the best mobile phase solvent system enabling better separation of compounds. The polar solvents used were; methanol, deionized water, acetonitrile, acetone, isopropyl alcohol and glacial acetic acid (protic solvent), while non-polar were hexane and intermediate ethyl acetate. Different combinations and ratios were mixed volume by volume and used on TLC plates. The best solvent system was then used in column chromatography packed with silica gel (60-200 mesh) to purify betanins in the extract for further analysis.

Extracted sample (1 mg) was spotted by using capillary tube on the TLC silica gel 60 (Merck, Germany) and labeled in pencil at the hash mark. The TLC plate were taken out until it reaches 75% on the top of the plate, then placed into UV light chamber (365nm and 254nm) to observe. The obtained color circled using pencil and their distance travelled were recorded to calculate Rf value (retardation factor) using the Equation (3.3).

$$Rf = \frac{\text{distance spot travels (cm)} * 100}{\text{Distance solvent travels (cm)}} \qquad \text{Equation (3.3)}$$

The solvent system with highest Rf values was then deduced as the best solvent system. This was used in column chromatography packed with silica gel (60-200 mesh) to purify betanins in the extract for further analysis.

3.13 UV-Vis Spectroscopy Analysis and Total Betalains Content

UV-Visible spectral analysis were used to identify the chromophoric groups present in dye molecules which are responsible for enhanced chemical interactions (Bukhari *et al.,* 2017; Rather *et al.,* 2016a, 2016b). Concentrated *B. vulgaris* peels and pomace extract and Reactive Orange HER (each 1 gram) was diluted with distilled water (100 mL) followed by serial dilutions (Appendix 3), and an aliquot was then introduced in a quartz cell (1 cm pathway). Measurements were carried out at wavelength of 400-800 nm using UV-visible spectrophotometer. The absorbance values were recorded for development of absorbance curves and quantification of betalains by applying Equation (3.4) according to Singh, Ganesapillai, & Gnanasundaram (2017) and Sturzoiu *et al.* (2011). The quantity was expressed as mg betalains/ 100 g

$$\text{Total betalains content (mg / 100 g)} = A \times DF \times MW \times 1\,000 / €L \qquad \text{Equation (3.4)}$$

Where; A is the Absorption value at wavelength of maximum absorbance (λ_{max}) ; DF is the dilution volume; L, path length of cuvette; MW, molecular weight of betalain(550g/mol) and €, the extinction coefficient for betalain (60000 L/mol).

Calibration curves and absorption spectra of the samples were generated to give an idea on the presence of color bearing compounds within the wavelength range (Saxena *et al.,*

2012; Espinosa *et al.,* 2012). The wavelength values corresponding to the uppermost absorbance values were recorded and graphs of absorbance against wavelength plotted.

3.14 Fourier Transform Infrared Spectroscopy

The purified and concentrated *B. vulgaris* extract was analyzed for the functional groups present, using FT-IR according to Huck (2015). Approximately 5mg of each sample and 5 mg KBr in each sample were used, and KBr in this was a standard. The FTIR spectrophotometer was operated at a spectral range of 350 -7500 cm^{-1} and a resolution of 8 cm^{-1}. The spectra were generated by OPUS version 7.5 Software and were interpreted using the guidelines as described by Kumar & Su-Ling Brooks (2018).

3.15 HPLC-UV and LC-MS Analysis

High Performance Liquid Chromatgography (HPLC) was carried out with modifications by using the Shimadzu Class HPLC System with a UV/VIS detector (Slatnar *et al.,* 2015). Ultra High Performance Liquid Chromatgography (UHPLC) system compatible with mass spectrometry was ideal for betalain screening in *B. vulgaris* peels and pomace pure extract. The LC-MS system was purposely used for confirmation of the identity of the betanin compounds through the mass spectral data interpretation, thereby approving or disapproving the HPLC-UV findings.

These methods provided for separation, identification and quantification as, they would form a strong evidence on betalains/betanin presence in *B. vulgaris* peels and pomace.

3.3.1 Standard Compound

Betanin (red beet diluted with dextrin) standard (betanidin 5-β-D-glucopyranoside), quantity 5mg, CAS number 53158-73-9 and lot number CFS201901 with percent purity

>= 95% was obtained from ChemFaces (Wuhan ChemFaces Biochemical Co., Ltd, China). This commercially purchased standard had a certificate of analysis containing information such as percent purity, residual solvents, physical description, quantity, storage conditions and shelf life.

3.3.2 HPLC-UV and LC- MS Conditions

The purified plant extracts were analysied by the HPLC equipped with UV detector at 538 nm and Supelcosil LC-18 column, under the conditions; pressure at 107 bar, mobile phase (isocratic) acetonitrile 70% and ammonium acetate buffer (0.7 mM) in water (30%), flow rate of 0.4 mL/min and injection volume of 10 µL. Column temperature was maintained at 35 °C. All the standard and sample solutions were filtered through a 0.45 µm nylon syringe filter (Millipore, USA). In this case, the instrumental software (Shimadzu Lab Solutions Version 5.60SP2) was used for data analysis and to generate chromatograms.

LC-MS analysis was carried out on Thermo Scientific TSQ Altis fitted with an OptaMax NG and coupled to a Vanquish Horizon UHPLC. UHPLC method was applied with the conditions: 0.1% formic acid in H_2O as solvent A and 0.06% formic acid in MeOH as solvent B, an isocratic gradient of 10% B for 10 min, 10%–100% B for 30 min, 100% B for an additional 10 min, using a flow rate of 0.3 mL/min; 5 µL injection volume and MS detector. The separation was carried out on a Thermo Scientific Hypersil GOLD aQ™ column (50 x 2.1 mm, 1.9 um) and the range for MS acquisition was *m/z* 50–1800. A capillary voltage of 4500 V, nebulizer gas pressure (nitrogen) of 2 (1.6) bar, ion source temperature of 200 °C, dry gas flow of 9 (7) L/min, and spectral rates of 3 Hz and 10 Hz for MS1 and for MS2 respectively. To acquire MS fragmentation, the 10 most intense

ions per MS1 were selected for subsequent collision-induced dissociation (CID) with stepped CID energy applied. The MS parameters were employed according to Garg *et al.* (2015). Sodium formate was used as an internal calibrant, and 2, 2-difluoroethoxy phosphazene (Fisher Scientific, USA) as the lock mass. All the data was processed through the TraceFinder version 4.1 software.

3.3.3 Standard Preparation

The commercially available betanidin 5-β-D-glucopyranoside was used as standard for betanin (red beet extract diluted with dextrin**)**. The standard (0.1 mg) was diluted in MeOH solution (100 mL), and used as standard stock solutions for generating calibration curves. The solution was stored at 4 °C following preparation.

The matrix test was performed to evaluate the suppression or enhancement of the ionization of analytes and the internal standard by the presence of matrix components in the samples in LC-MS. Following the sample preparation method, samples were prepared to re-dissolve the solution step; 200 µL of 1.5 ng/mL of betanidin 5-β-D-glucopyranoside and 25 ng/mL of the internal standard were then added.

3.3.4 Sample Preparation

The pure extract in powder (0.1g) was dissolved in MeOH (100 mL). An aliquot of 1 mL was weighed in a 10-mL volumetric flask, and then the volume (10 mL) was made up with 2% HCl and MeOH mixture. The sample was then swirled to mix thoroughly. This was followed by filtration using 0.45 µm nylon syringe into HPLC amber vials. Triplicate samples were prepared for HPLC-UV and LC-MS analysis.

3.3.5 Method Validation/Quality control

3.15.1.1 Repeatability

The repeatability of the results were ascertained by the close retention times of the standard betanidin 5-β-D-glucopyranoside solution ranging from the five concentrations 10 ppm, 15 ppm, 20 ppm, 27 ppm and 35 ppm in triplicates at different times of day 1 and same triplicate concentrations at different times (day 2), for intra-day repeatability (same day) and different day (inter day) precision. Their respective retention times and peak areas were tabulated accordingly.

3.15.1.2 Calibration Curve Linearity

From the stock solution, 1 mL aliquot was diluted to 10 ppm, 15 ppm, 20 ppm, 27 ppm and 35 ppm solutions and injected to generate a five point calibration curve for the standard compound. The correlation coefficient, R^2 value was 0.9928. It is greater than 0.96 predicting good results.

For LC-MS, the analytical curve was constructed using values ranging from 0.02 ng/mL to 50 ng/mL. Calibration curves were obtained by weighted (1/concentration) least-squares regression analysis and fitted to Passing and Bablok curve. Calibration curve standards and samples were prepared for analysis in duplicates. The correlation coefficient, R^2 value of Passing and Bablok curve was 0.9988 that was also greater than 0.96, hence predicting realiable results. Precision and accuracy for the interpolated concentrations of the calibration points were maintained within ± 15% of their nominal values.

3.15.1.3 Sensitivity

Sensitivity of the method was measured in terms of Limit of detection (LOD) and limit of Quantification (LOQ). The LOD and LOQ were determined by measuring the signal to noise ratio from low concentration of standard analyte and comparing with baseline peak of blank sample. Triplicate 1 ppm, 0.8 ppm, 0.6 ppm, 0.4 ppm and 0.2 ppm samples of the batanin standard were injected onto the HPLC-UV and LC-MS. A calibration curve was developed to obtain the regression line standard deviation and the slope of the curve. The LOD was determined as the lowest concentration at which a peak was detected corresponding to the expected retention time and is normally taken to be three times the noise level. LOD was established by applying the Equation 3.5.

LOQ was set within ± 20% in LC-MS analysis. The LOQ was estimated as the minimum concentration at which the peaks could be successfully quantified in accordance with the baseline noise method. It was estimated at a signal to-noise ratio of five (5). The LOQ was experimentally determined by administering six injections of the standard at the LOQ concentration. The signal-to-noise ratio was calculated by selecting the noise region very near to the signal peak that was at least 10 times the width of the signal peak at half its height and applying Equation 3.6.

$$LOD = 3S_a/b \qquad \textit{Equation (3.5)}$$

$$LOQ = 10S_a/b \qquad \textit{Equation (3.6)}$$

where S_a is the regression line standard deviation and b is the calibration curves slope.

3.3.6 Identification of Betanins by HPLC and LC-MS/MS

One of the objective of this study was aimed at qualifying the main peak observed in *B. vulgaris* using HPLC and LC-MS by comparing it with the commercially available

betanin standard. The chromatograms of the three sample injection into HPLC were used to calculate the concentration as the unknown sample, by applying their peak areas, average concentrations and taking into consideration their standard deviation. The compounds were further identified by searching the accurate mass and the MS data using MetFrag (Wang, Zhang, Yan, Han, & Sun, (2014); Wolf, Schmidt, Müller-Hannemann, & Neumann, 2010) in the LC-MS. The MS peaks and their intensities were imported into MetFragBeta and the neutral exact mass of the parent ions were calculated and their masses searched within 5 ppm using PubChem databases.

3.3.7 Quantification of Betanins

The betanin content were quantified from HPLC-UV data using the external standard (ESTD) method of quantification. The amounts of betanin (mg/g) contained in the plant sample was expressed as $(A \times DF) \times CF/S\ Wt$; where A = mg/mL amount of betanin expressed as the external standard equivalent (betanidin 5-β-D-glucopyranoside) from the calibration curve; *DF* = dilution factor (*DF* for the plant sample was calculated as initial weight of sample (g) + 10 g dilution solution/weight of 1 mL aliquot (g) × 10 mL; *DF* for *B. vulgaris* peels and pomace powder samples was 10 mL); *S Wt* = initial sample weight (g); and *CF* = molecular weight correction factor to convert the betanins (mg/g) calculated as the external standard equivalents to their respective forms (Slatnar *et al.*, 2015).

3.16 Optimization of Dyeing Parameters on Cotton Fabrics

The optimized extraction conditions were used to prepare the dye bath solution to impart colour on cotton fabrics. The warp count, weft count, ends/inch, picks/inch, GSM and absorbency of the scoured and mercerized woven pure cotton fabric were firstly

considered before dyeing. The absorbency rating of the scoured and mercerized fabrics was determined by using spray master with American Association of Textile Chemists and Colorists (AATCC) certified spray Test Rating Chart.

RSM and CCD type of design (Hamanthraj *et al.*, 2014) was applied for optimization of dyeing process variables. Table 3.3 shows the respective uncoded factors; dyeing temperature, (^{o}C)-A, dye bath time (minutes)-B and pH-C. Twenty experimental runs were designed (Table 3.4). The factors chosen that affects percentage exhaustion of dye were; temperature, dyeing time and pH of the dye bath. In the dyeing optimization, natural mordant concentration (order of tannic acid-alum) of 50% **(**o.w.f.), M: L ratio (1:20 o.w.f) and 2g pure cotton fabric were kept constant while employing post-mordating method.

Table 3.3. Experimental variables and their levels for dyeing.

Variables		Coded levels				
Symbol	Name	$-\alpha$	-1	0	+1	$+\alpha$
A	Temp.(^{o}C)	2.155	20	55	90	112.16
B	Time(mins)	1.515	30	75	120	148.49
C	pH	2.4175	4	6.5	9	10.5825

Table 3.4. Experimental runs design table (randomized) for dyeing.

Run	A	B	C
1	-1.000	-1.000	1.000
2	-1.000	1.000	1.000
3	0.000	0.000	0.000
4	0.000	0.000	0.000
5	1.000	1.000	-1.000
6	0.000	0.000	0.000
7	0.000	0.000	0.000
8	1.000	-1.000	1.000
9	1.000	-1.000	-1.000
10	-1.000	-1.000	-1.000
11	1.000	1.000	1.000
12	-1.000	1.000	-1.000
13	1.633	0.000	0.000
14	0.000	0.000	0.000
15	0.000	0.000	1.633
16	0.000	0.000	0.000
17	-1.633	0.000	0.000
18	0.000	1.633	0.000
19	0.000	0.000	-1.633
20	0.000	-1.633	0.000

3.17 Statistical Modelling of Dyeing Variables

For an experimental design with three factors, the model encompassing linear, quadratic and cross-terms can be expressed as Equation (3.2). Relative change in colour strength (ΔE) was used as the response (y). Multiple linear regression (MLR) using MS Excel was performed to obtain the coefficient values. The response surface plots were obtained through Minitab statistical process modelled through CCD. Response surface methodologies graphically illustrate relationships between variables or parameters and responses. In this way, it is possible to obtain optimum parameters or conditions of an experiment. Statistical significances of the predicted models were evaluated by ANOVA and least squares techniques generated to determine which factors significantly affect the studied response variables. *P*-value represents a decreasing index of the reliability of a result such that a p-value less than 0.05 (typically ≤ 0.05) is statistically significant implying that there is a strong evidence against the null hypothesis. This is an indicator that there is less than a 5% probability the null is correct and that the results are random.

3.18 Dyeing Procedures for Cotton Fabrics

The extracted dye from each extraction process was applied on the scoured and mercerized cotton fabric samples in order to evaluate their dyeability. Dyeing method reported by Hong (2018) was performed in a laboratory scale water bath dyeing machine at pre-optimized dyeing conditions. Preparation of dye stock solution was done as described by Bukhari *et al.* (2017). The control measures were un-mordanted fabrics and reactive dyeing (Reactive Orange HER) on cotton fabrics. Reactive dyeing was done following the standard method; Exhaust dyeing at water bath temperature of 80 °C, and dyeing time of 45 minutes). Additionally, sodium sulphate (Glauber's salt) is added (40g/L) 10 minutes before ending the dyeing process of reactive dyeing to aid in fixation, before finally adding 2g/L of soda ash to raise the pH.

The pH in the *B. vulgaris* peels and pomace natural dyeing was controlled by solution of hydrochloric acid (to lower pH) and solution of sodium hydroxide (to raise the pH). The dyed cotton fabrics were then washed with distilled water to get rid of excess dye molecules at surface of cotton fabrics (Ding & Freeman, 2017). Wetting agent, penetrol was added to improve dye solubilty and absorption, and 20 % o.w.f sodium sulphate was added in the process for maximum dye exhaustion. The samples from each runs were washed in 2 g/L sodium Lauryl Sulphate (SLS) to remove excess dye particles on the fabrics.

3.19 Mordanting Procedures

Mordanting were then performed following optimized dyeing conditions using natural mordants and metallic mordants at varying concentrations (20-50% o.w.f) following the three methods of mordanting; pre-mordanting, simultaneous and post mordanting

methods (Rather *et al*., 2016b). The mordants were; natural mordants (order of tannic acid -alum) and metallic mordants (iron (II) sulphate, sulphate, copper (II) sulphate and potassium dichromate). The dye concentration (70% o.w.f), and fabric weight (2 grams) were kept constant. To achieve maximum dye exhaustion, 20 % o.w.f sodium sulphate was added into the dye bath (Prabhavathi *et al.,* 2014).

In pre-mordanting, the mordant was first added into a beaker containing the fixed quantity of dye solution, followed by addition of cotton fabric samples. The whole mixture was thereafter slowly brought to boil in the water bath. After dyeing, the solution was allowed to cool, the fabric gently removed, washed, air dried and stored for analysis.

Simultaneous mordanting involved dyeing and mordanting at the same time while post mordanting involved addition of the mordant after dyeing. The mordant was added to the dye bath in the final ten to twenty minutes of simmering. The fabric was ejected out of the solution, left to cool, normal washing performed to remove unfixed dye, then finally air drying awaiting colour strength analysis.

3.20 Colour Measurement

The dyed and naturally mordanted fabrics colour strength were accessed by observing in spectra vision (5L) color matching booth using daylight, D65 and applying Bench-top spectrophotometer. Undyed (white) fabric was used as a blank or reference material. The obtained relative colour strength (ΔE) values were used as response (*y*) in dyeing regression analysis.

The relative colour change in strength (%), reflectance (R %), colour strength (K/S value) and the characteristic CIELab color coordinates, that is (Light/darkness (L*), tones (a*

& b*) and ΔE*ab of the dyed samples were determined by the spectrophotometer color meter (Khan *et al.* 2018a, 2018b). The colour measurements were undertaken by taking the *B. vulgaris* peels and pomace dyed specimens 10 cm square against undyed specimens separately placed on a horizontal platform with the measurement head and the sample being as close as possible or in contact for precise measurement. Both unmordanted and mordanted natural dyed cellulosic fabrics were automatically illuminated under D_{65} at 10° as described by Rather *et al.* (2016b).

From the reflectance (R %) values obtained, the color strength (K/S) in the visible region of the spectrum between 400–700 nm was calculated based on the Kubelka–Munk Equation 3.7.

$$\frac{K}{S} = \frac{(1-R)^2}{2R} \qquad \textit{Equation (3.7)}$$

Where K, S and R are the absorption coefficient, scattering coefficient and reflectance of the dyed samples respectively.

Chroma (c*) and hue angles (h°) were calculated using the Equations 3.8 and 3.9 respectively:

$$Chroma = (a^{*2} + b^{*2})^{1/2} \qquad \textit{Equation (3.8)}$$

$$Hue = (h = tan^{-1}\, b^*/a^*) \qquad \textit{Equation (3.9)}$$

Chroma (C*) is the value of the distance from the lightness axis (L*) and C*starts at zero in the center. Hue angle starts at the +a* axis and is expressed in degrees (e.g., 0° is +a*, or red, and 90º is +b, or yellow). The L*, *a**, *b** values and delta values of the three axes of the CIELab i.e. ΔL*, Δa*, Δb* and ΔE* were observed and recorded. Total

colour change (ΔE) of the dyed fabric were calculated by the spectrophotometer color meter basing on Equation 3.10:

Total Colour change (ΔE):

$$(\Delta E) = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad \textit{Equation (3.10)}$$

Where,

ΔL* = L* sample - L* standard, Δa* = a* sample - a* standard and Δb* = b*sample - b*standard.

From the HSV color space, red, orange and yellow for instance have hue (h^o) angles of 0°, 30° and 60° respectively (Khan *et al.*, 2018b).

3.21 Colour Strength Equivalence, Ceq

Color strength equivalence represents the color intensity of extracted dye solution compared to a synthetic dye. Natural dyes commonly have pale shade depths in dyed materials and these were clearly observable in spectra vision color matching booth using daylight, D 65. This is related to the color strength of the dye extract. Color strength equivalence for the *B. vulgaris* peels and pomace natural dye as compared to commercial reactive dye, R. orange HER (C.I. 84, C.I. No. 258200) was determined by using the approach applied by Abera Kechi (Kechi, Chavan, & Moeckel, 2013). The absorbance of aqueous extract (Aextr) of the dye plant was measured at the wavelengths of maximum absorption (λmax) using DU 720 UV-Vis spectrophotometer. The absorbance (Aextr) for the dye plant solution was measured five times and absorbance repeated three times in each case and the average results reported. Care was taken to keep the absorbance value within two by suitably diluting the known volume of dye extract. The Aextr was

multiplied with corresponding dilution factor (DF) during calculation of equivalent color strength compared to R. orange HER, using Equation (3.11).

$$Ceq = \frac{Aextr}{\varepsilon RO84d} \qquad \textit{Equation (3. 11)}$$

Where Ceq is the color strength equivalence of Reactive Orange HER (g dm^{-3}); Aextr is absorbance of dye plant extract; ƐRO84 is the extinction coefficient of Reactive orange HER (7.94 dm^3 cm^{-1} g^{-1} at λ=491nm) and d is the path length of cuvette (cm).

The colour strength of synthetically dyed cotton was ascertained by dyeing the cotton fabric sample with commercial dye (Reactive Orange HER) following standard dyeing conditions for exhaust reactive dyeing; 0.375 g/L dye, dyeing time of 45 minutes at temperature of 80 ºC. The quantity used (0.375 g/L) is equivalent to 70% o.w.f natural dye used on the fabric as per Equation 3.11. The colour strength was then obtained using Bench-top Spectrophotometer and the values compared with those of naturally dyed fabrics. Mass equivalence (meq) was then ascertained by multiplying the obtained Ceq with 20, since dyeing ratio was 1:20 (Kechi *et al.,* 2013).

3.22 Colour Fastness Properties Tests

The samples were pre-conditioned before testing as per the international standard organization (ISO) standards. The test specimens were kept in standard laboratory conditions of relative humidity, 65% $\pm$ 5 and temperature of 22-25 °C for 24 hours. The colour fastness of all the dyed fabric samples were evaluated according to ISO standards and procedures which are; ISO 105-C-06:2010 (E) for colour fastness to domestic and commercial laundering; ISO 105 Part X12 for colour fastness to rubbing; ISO 105-B02

for colour fastness to artificial light - Mercury blinded tungsten lamp (MBTL) fading lamp test and ISO 105 E04 for perspiration fastness.

The standard reference samples and dyed cotton samples were then assessed using grey scales for color change and staining, according to ISO/AATCC standard methods and ratings. The greyscale consists of nine pairs of grey color chips each representing a visual difference and contrast as; 1:1/2:2:2/3:3:3/4:4:4/5:5. The fastness rating goes from 1 to 5, where 1 means: Large visual change (Worst rating), Poor; 1/2: Fairly moderate to moderate; 2: Moderate; 2/3: Fairly good, to good; 3: Good; 3/4: Good to very good; 4: Very good; 4/5: very good to excellent; 5: No visual change (Best rating) –Excellent.

3.22.1 Wash Fastness

Fastness to washing was tested for *B. vulgaris* peels and pomace natural dye against AATCC standard adjacent fabric samples following ISO 105-C-06 test procedures. The commercial R. Orange HER dyed specimens fastness test was also done for comparative analysis with the naturally dyed fabrics. Dyed specimens were cut into 5 x 10 cm sizes and washed in Paramount wash master Launder-o-meter. A detergent solution was prepared using ISO certified sodium oleate at concentration of 5 g/L in distilled water (total liquor volume of 150 mL). The prepared samples were then placed in stainless steel canisters along with ten steel balls and then fixed in the launder-o-meter. They were subjected to 5 cycles for 30 minutes with temperature maintained at 40 °C. The change in color of the specimens and degree of staining of the standard reference samples were assessed via the grey scales (according to ISO 105-C-06:2010-E).

3.22.2 Dry and Wet Rub Test

ISO 105 X12 test method was a guideline in performing this test. In dry rub test, the test specimen was cut about 7.5x10 cm from the *B. vulgaris* peels and pomace dyed cotton fabrics and the specimen was mounted horizontally on the Paramount crock meter using spring clip and 16 mm finger. The weight was adjusted to 9 ± 0.5 newton and the machine was operated for ten revolutions.

For wet rub test clean white crocking cloths (5 x 5cm) were immersed in cold water and excess water removed by inserting in between 10 cm by 10 cm blotting papers. They are then attached onto the spindle finger with spring clip and the dyed specimen mounted on flat acrylic specimen holder. The weight applied was 9 ± 0.5 newton for 10 revolutions. In both dry and wet rubbing, the color change and color transfer from test dyed specimen to crocking cloth (staining) was assessed via the greyscale for color change and staining according to ISO/AATCC standards.

3.22.3 Light Fastness

The resistance of the natural dye and commercial dye toward natural light were analyzed by exposing the side to be tested of the cotton sample with mercury-blended tungsten lamp for eight hours according to ISO 105 B02 method. The MBTL lamp correlates with xenon lamp and natural day light. The specimen strip (50 x 10mm) and one strip each of 1 to 8 Light Fastness Standards (LFS) was mounted on specimen card and the same is put on Type ‘A’ Rectangular cell. This was done along with double slot mask (1/3 area cover) with the mask 2/3 of the specimen was exposed to light. The exposed regions were inspected for colour change and this analysis provides information on degree of light

fastness. The fading of the cotton fabric with the fading of the commercial dye R. Orange HER and the change in color of the specimen assessed with the greyscales (IS-2454-85).

3.22.4 Perspiration Fastness

The control and naturally dyed cotton fabrics were tested for fastness to perspiration according to ISO 105 E04 method (acid and alkaline perspiration) using color fastness to perspiration tester. To prepare an alkaline artificial perspiration solution, 0.5 g of L-histidine monohydrochloride monohydrate ($C_6H_9O_2N_3 \cdot HCl \cdot H_2O$), 5 grams of sodium chloride (NaCl) and 2.5 g of Sodium dihydrogen orthophosphate anhydrous (Na_2HPO_4) were measured and made to one liter with distilled water and adjusted to pH 8.0 $\pm$ 0.1 with 0.1 N sodium hydroxide. Acidic solution was freshly prepared by mixing 0.5g L-histidine mono-hydrochloride monohydrate, 5 g sodium chloride, 2.2 g sodium dihydrogen ortho phosphate and distilled water to make one litre solution. The pH was brought to 5.5 with 0.1 N sodium hydroxide.

Two undyed cotton specimen each 6 × 6 cm were used as sample. The *B. vulgaris* peels and pomace dyed specimen were placed between the two pieces of white cloth and sewn along one side to form a composite sample. One composite sample was immersed in a solution of pH 8.0 at the liquor ratio of 20:1 and allowed to remain in this solution at room temperature for 30 min. The solution was poured off and the composite sample placed between two glasses plates (75 × 65 mm) of ISO perspirometer testing machine under a force of about 4.5 kg. The other sample was treated in the same way but with the solution at pH 5.5. The apparatus containing the samples were then placed in an oven for 4 hours at 37 ± 2 °C after which the sample was separated from the white cloth and dried

separately in air at the temperature not exceeding 60 °C. The change in colour of the specimen and the staining of the white cloth were assessed via the greyscale.

3.23 Physical and FT-IR Characteristics of Dyed Cotton Fabrics

The mordanted *B. vulgaris* peels and pomace dyed cotton fabrics were then observed under D65 (daylight) colour matching cabinet to analyse the depth and hue of the colours. A table of the observed fabics was then created reflecting on the dyed and differently mordanted fabrics.

FT-IR analysis of bleached cotton fabrics and optimumly dyed and mordanted cotton fabrics were performed to observe the effects of dye and mordants on the fabrics spectroscopically. Bleached and the naturally dyed fabrics were each grinded separately to fine powder and approximately 5 mg of each sample collected on Alpha Bruker Optic instrument and analysed separately. Approximately 5 mg KBr in each sample were used. The FT-IR spectrophotometer was operated at a spectral range of 350 -7500 cm^{-1} and a resolution of 8 cm^{-1}. The spectra were generated by OPUS version 7.5 Software and were interpreted using the guidelines as described by Kumar & Su-Ling Brooks (2018).

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Evaluation on Optimization of Extraction Conditions of the Natural Dye

The natural dye was extracted under fixed conditions of temperature 68 ^{o}C using 20 grams of plant powder and varying time and M: L ratios (i.e. plant powder to methanol solvent). For the extraction of *B. vulgaris* peels and pomace dye, the experimental matrix is shown in Table 4.1.

Table 4.1. Experimental matrix for extraction yield.

Runs	Solvent ratio ,mL(M:L Ratio)	Extraction time(hrs)
1.	200	7.5
2.	400	5
3.	100	10
4.	400	10
5.	100	5
6.	80	7.5
7.	200	4
8.	200	11

Fitting the second order polynomial Equation 3.2 and expressing response, y as betanin content and positioning it as a function of independent variable while using time (A) of extraction and M: L ratio (B), a linear model was obtained as;

$$y = 193.15+13.66A+ 1.11B \qquad \textit{Equation (4.1)}$$

4.1.1 Effect of Time and M: L Ratio on Extraction Yield

The respective extraction yield calculated using Equation 3.) are listed in Table 4.2. There is a direct correlation between extraction yield, the betanin content and by extension variables affecting extraction. The longer the time of extraction, the higher the

extraction yield or betanin content and vice versa (Figure 4.1). A combination of methanol and formic acid water (50:5/vv) allowed complete extraction and stability of flavonoids as well as preventing degradation of colour in plants (Rosa, Garcı´a-Falco´n, Simal-Ga´ndara, Rodrigues, & Domingos, 2010; Manasa *et al.*, 2013).

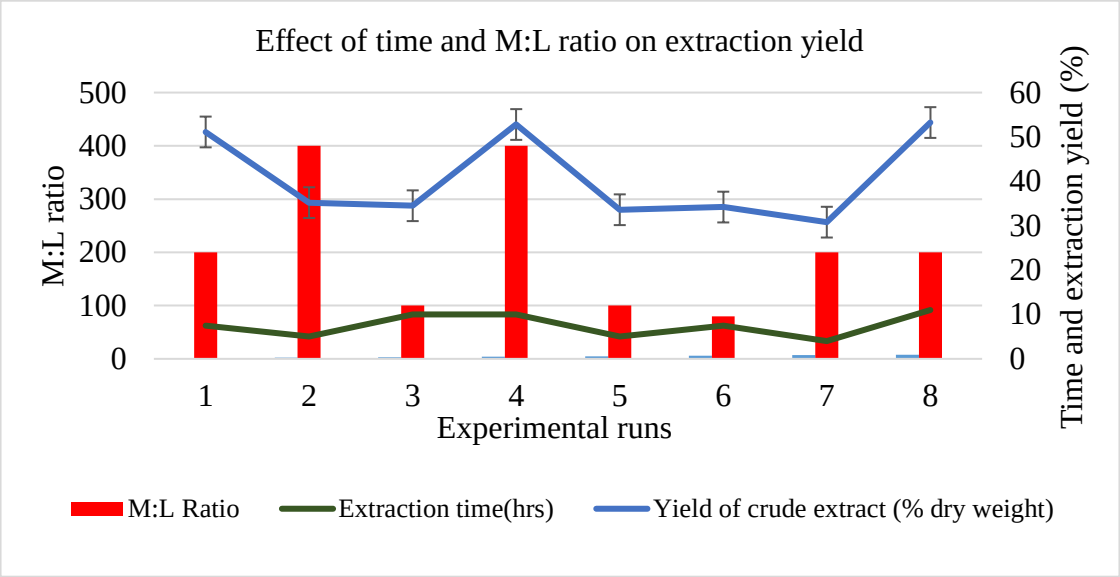


Figure 4.1. Effect of extraction time and M: L ratio on % extraction yield.

Working out Pearson correlation between yield and extraction time gives a correlation coefficient (ρ) of 0.684, which is greater than zero means that there is a positive relationship between yield and extraction time (Table 4.2). Correlation coefficients (ρ) are indicators of the strength of the linear relationship between two different variables, x and y. A linear correlation coefficient that is greater than zero indicates a positive relationship while that is less than zero signifies a negative relationship. A value of zero indicates that there is no relationship between the two variables x and y. The possible values of the correlation coefficient is in the range of -1.0 to 1.0. That is, the values

cannot exceed 1.0 or be less than -1.0. A correlation of -1.0 suggest a perfect negative correlation, while a correlation of 1.0 suggest a perfect positive correlation.

Table 4.2. Yield of crude extract from each variable in experimental runs.

Runs	Amount of solvent(ml)	Extraction time (hrs)	Yield of crude extract (% dry weight)
1	200	7.5	51.12 ± 1.24
2	400	5	35.21 ± 2.56
3	100	10	34.53 ± 1.36
4	400	10	52.81 ± 1.15
5	100	5	33.60 ± 1.87
6	80	7.5	34.23 ± 1.32
7	200	4	30.84 ± 0.98
8	200	11	53.25 ± 1.12
Results presented as mean values of three replicates ± SD			
ρ =0.684485(extraction yield vs extraction time)			

Equally, the extraction yield is relative to the betalain content (Table 4.3). Betanine (belonging to betalain group) is the main compound present in high quantities in the red *B. vulgaris* plant deriving its red colouring (Kathiravan *et al.*, 2014). Thus, the higher the extraction yield the higher the betalain content and vice versa.

Table 4.3. Percent yield of crude extract with respective quantity of betalains.

Extraction yield (%)	Extraction time (hrs)	Amount of solvent(ml)	Quantity of Betalains (mg / 100 g)
51.12 ± 1.24	7.5	200	375.12 ± 2.25
35.2 ± 2.56	5	400	311.10 ± 3.34
34.5 ± 1.36	10	100	297.92 ± 4.42
52.8 ± 1.15	10	400	362.31 ± 2.89
33.6 ± 1.87	5	100	302.50 ± 3.16
34.2 ± 1.32	7.5	80	357.52 ± 1.78
30.8 ± 0.98	4	200	307.10 ± 4.47
53.25 ± 1.12	11	200	371.32 ± 3.92
Results presented as mean values of three replicates ± SD			
ρ =0.832353746 (Extraction yield vs Betalain content)			

Large quantities of solvent with less time limit interaction of the solvent with plant molecules resulting in low betalain yield (Figure 4.2). This is true from the correlation coeffcient between extraction yield and betalain content calculated below of 0.83 which is closer to 1, showing a positive correlation between the mentioned variables.

By way of ANOVA (Table 4.4) the R^2 value shows there was a moderate positive linear relationship (Equation 4.1) among the variables hence the regression model fits the data. That is 52% of variance in betalain content was attributed to the variables. The slope (b_0) was 193.15 depicting that the expected change of response, y by one unit change of variables is immense.

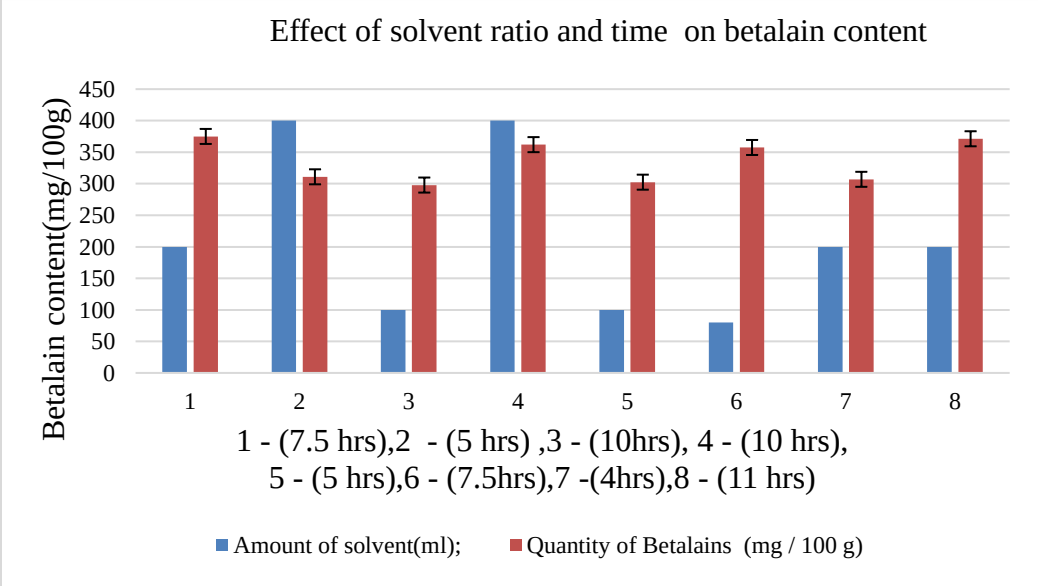


Figure 4.2. Comparison of Time (hrs) and M: L ratio with quantity of betalains.

The significant F value is less than 0.05. Therefore, the relationship is significant. Time has significance to extraction as observed from the p-value ($p<0.05$) while M: L ratio (amount of plant material to solvent) $p>0.05$ demonstrating it as a weak evidence or

insignificant to extraction meaning other factors in the solvent could be contributing, such as polarity. The slope (b_0) has low p value (<0.05) meaning it's different from zero. This generated optimized extraction conditions in Table 4.5.

Table 4.4. ANOVA for dye extraction.

Properties	df	SS	MS	F	Significance F	P-value
Intercept	2	6533.2761	3266.638	6.1545	0.01608	0.00022
A (Time, hrs)	11	5838.5010	530.7728			0.00641
B (M:L ratio)	13	12371.777				0.32811
Regression Statistics						
Multiple R	0.72669					
R^2	0.52808					
Adjusted R^2	0.44228					
Standard Error	23.0385					

Table 4.5. Optimized extraction conditions.

Parameter	Optimized conditions
Amount of solvent(M:L)	1:10
Extraction time(hrs)	11

4.2 Phytochemical Constituents of *B. vulgaris* Plant

The chemical constituents of methanolic extract of red beet roots was proved to contain phenolics, flavonoids , tannins , alkaloids , anthocyanin and carotenoids compounds as seen practically in Appendix 5 and results in Table 4.6. These results corroborates to previous studies done by Mroczek *et al*.(2012); Odoh & Okoro (2013), and Mokhtari-Dehkordi, Hojjati, Rouhi, Rabiei, & Alibabaei (2014) who found that the main components of red beet root extract are alkaloids, amino acids, flavonoids and polyphenols. The phytochemical components are responsible for both pharmacological and toxicological activities in plants (El-Beltagi, 2011). The *B. vulgaris* peels and pomace red to violet colour is attributed to water-soluble pigments betalains (betacyanins that are red-violet color). This was evidenced by its colour and the UV-Vis absorbance

maxima of betalain at 538 nm. This research also established that *B. vulgaris* peels and pomace contains carbohydrates, proteins and saponin. This establishment went in line with 26 triterpene saponins that were characterized in beetroots whereby 17 of them had not been previously reported thus 7 triterpene saponins were identified as new compounds (Mikolajczyk-Bator & Pawlak, 2016).

Table 4.6. Summary of phytochemical constituents in *B. vulgaris* peels and pomace extract.

S/No.	Test	Methanolic extract
1.	Carbohydrates(Benedict's test)	++
2.	Proteins(Biuret test)	++
3.	Amino acids, tyrosine(Million's test)	++
4.	Sterols and triterpenes (Salkowaski test)	+
5.	Cardiac glycosides (Keller killiani test)	-
6.	Saponin glycosides (foam test)	+
7.	Flavonoids (Ferric chloride test)	++
8.	Tannins (Ferric chloride test)	+
9.	Alkaloids (Wagner‟s reagent)	+++

Key: '+++' (abundant); '++' (present); '+' (trace amounts); '-'(not detected).

4.3 Characterization of *B. vulgaris* Extract

The characterization of the *B. vulgaris* peels and pomace plant extract was performed by use of UV-Vis according to Bukhari *et al.* (2017) and Rather *et al.* (2016a, 2016b), FTIR analysis following guidelines according to Huck (2015), HPLC-UV analysis at 538 nm for compound separation, identification and quantification as previously described by Nemzer *et al.* (2011) and LC- MS technique to further elucidate the structure and support HPLC-UV output according to Kumorkiewicz (2017).

4.3.1 UV-Vis Spectroscopy Analysis and Betanin Content Estimation

The absorbance values of the extract after serial dilution and analyzing in UV-Vis were used to generate a calibration curve (Figure 4.3). The UV-Visible spectrum of *B. vulgaris*

plant extract and that of R. Orange HER is presented in Figure 4.4 with λ_{max} at 538 nm and 492 nm respectively. The higher wavelength of absorption is called wavelength of maximum absorption, which is denoted λ_{max}. The strong absorption band at 538 nm is attributed to the betanin and this finding is similar to that of Espinosa *et al.* (2012). Absorbance bands at 538 nm are characteristic for red violet betalain group, betacyanin (Singh *et al*., 2017). The absorption and emission of radiant energy from the UV–Visible spectrophotometer light source initiates $\pi \rightarrow \pi^*$ transition in aromatic rings (alternating conjugates of double bond to single bond) of the *B. vulgaris* peels and pomace dye molecule (auxochrome). Similarly, absorption and emission of radiant energy lead to $n \rightarrow \pi^*$ electronic transition in heteroatom of the dye molecule (chromophore). Hence, combination of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions in the dye molecules resulted in color emission at 492 and 538 nm respectively.

R. Orange HER dye has a shoulder (Figure 4.4), indicating it is a non-homogeneous mixture. The absorption in the 400 nm to 800 nm region is attributed to various chromophores responsible for colour as well as conjugate systems (Saxena *et al.,* 2012; Espinosa *et al.,* 2012). Their close λ_{max} demonstrated that their colour intensities are also very close. However, there is a slight shift in the wavelenghth maxima compared to the synthetic one. This means the natural dye has relatively poor absorbance in the visible region but good absorbance in the ultra violet region. R. Orange HER belongs to double azo class hence the azo compounds such as napthalenes, benzenes and triazenes makes it have wavelength maxima at around 492 nm. Molecules having conjugated systems of π electrons absorb light closer to the visible region than saturated isolated double or triple bonds. Longer conjugated system causes increased wavelengths of the light being

absorbed. Additionally, the type of solvents influences the quality and shape of spectrum. A substance may absorb at a particular wavelength in one solvent but shall absorb partially at the same wavelenth on another solvent. Methanol and ethanol are among the suitable solvents in UV-Vis analysis as they do not absorb radiation above 210nm (shorter wavelengths); while solvents such as chloroform, dichloroethane etc can absorb above 240nm hence longer wavelengths.

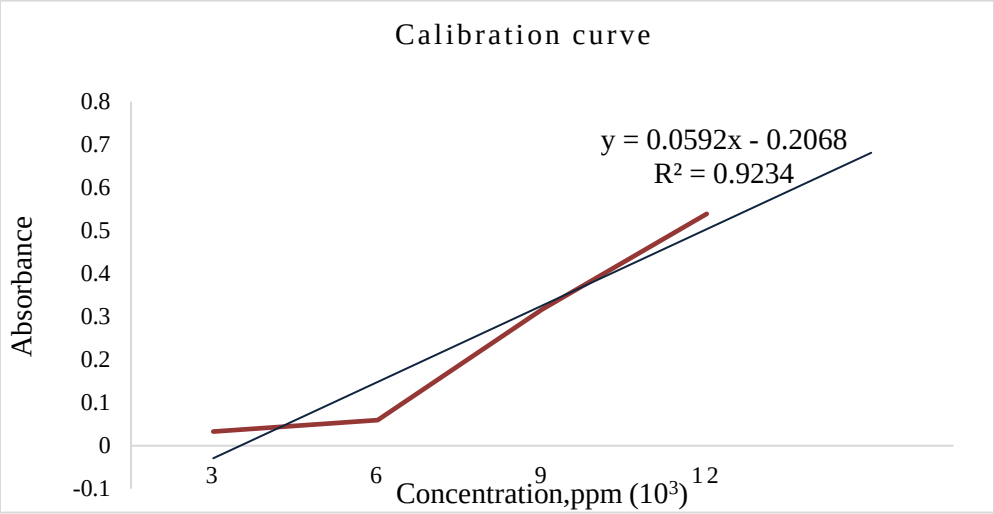


Figure 4.3. Calibration curve of *B. vulgaris* peels and pomace methanolic extract.

The betanin concentrations in the plant extract were calculated in comparison to the standard curve, described by the equation y = 0.0592x-0.2068 (R^2 = 0.9234) in Figure 4.3. The graph was made linear to agree with Beer-Lambert law as described by Uddin (2015). By applying Equation 3.4 to estimate the content of total betalains, the calculated values averaged at 335.25 ± 3.28 mg/100 g of dry weights as seen in Table 4.7. The result obtained were in accordance with Kechi *et al.* (2013), who found that, the total betalain content of red beet were 250 to 850 mg/100g by absorption.

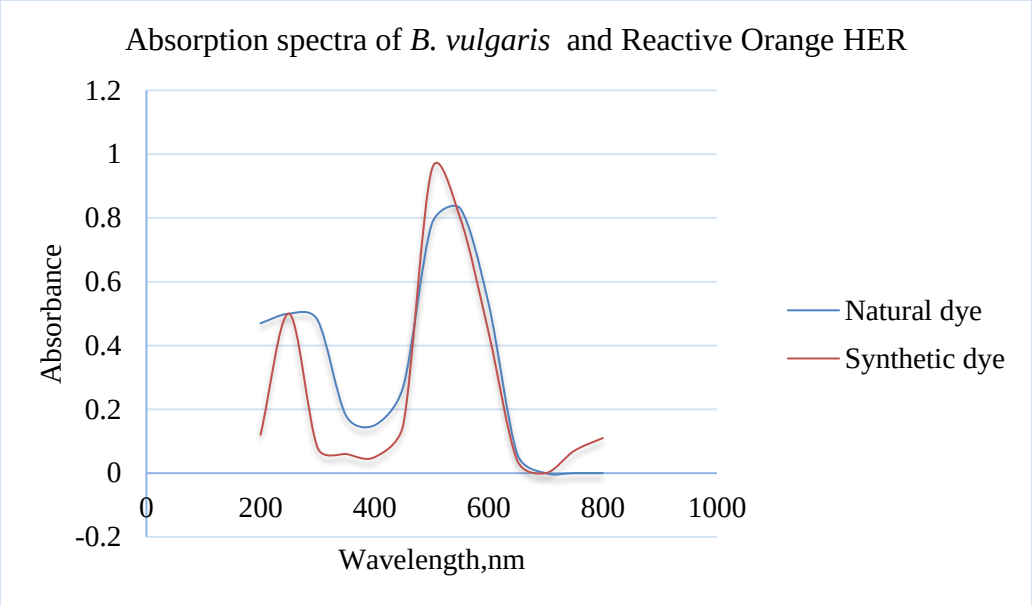


Figure 4.4. Absorption spectra of *B. vulgaris* peels and pomace extract and Reactive orange HER.

Table 4.7. Betalain content from absorbance data.

Runs	A max, 535-537nm	Quantity of Betalains (mg / 100 g)
1.	0.82	375.12 ± 2.25
2.	0.68	311.10 ± 3.34
3.	0.65	297.92 ± 4.42
4.	0.79	362.31 ± 2.89
5.	0.66	302.50 ± 3.16
6.	0.78	357.52 ± 1.78
7.	0.67	307.10 ± 4.47
8.	0.81	371.32 ± 3.92
Mean($\bar{x}$)		335.25±3.28
Results presented as mean values of three replicates ± SD		

4.3.2 FT-IR Analysis

Infrared spectroscopy (FTIR) analysis was performed to determine the functional groups and conformational characteristics of betanin molecule. The assignment of such functional groups was performed according to the available literatures.

Appendix 4 and Figure 4.5 show the FTIR spectrum of the KBr standard and B. *vulgaris* peels and pomace isolate respectively in the region of 4000–500 cm^{-1}. Different absorption bands characteristic of functional groups in the *B. vulgaris* peels and pomace methanolic extract were observed. From the left side, the band at 3282.82 cm^{-1} was attributed to the stretching vibration of the –OH bond probably due to hydroxyl groups (Kumar, Ritesh, Sharmila, & Muthukumaran, 2017). The band located at 2932.96 cm^{-1} was attributed to the vibrational stretching of the C -H bond due to alkanes (Molina *et al.*, 2014) while the band at 1588.91 cm^{-1} was assigned to the extension stretching vibration of the C = N bond. The C = N bond is the aldimine bond that binds the betalamic acid with the dopa cycle. Therefore, the presence of these nitrogen containing functional groups probably indicated the presence of betalains in the peels and pomace of *B. vulgaris* as they are N containing compounds. Results of the present study were in agreement with the findings of Kumar *et al*. (2017). The –OH and C–H bonds indicates the presence of hydroxyl groups such as alcohol, and different types of carbohydrates such as lignin, cellulose, hemicelluloses and pectin (Talari, Martinez, Movasaghi, Rehman, & Rehman, 2017) respectively. The next band at 1342.83 cm^{-1} correspond to the aliphatic bending of the C–H bond of the organic compound (Molina *et al.*, 2014). The band at 1045.38 cm^{-1} was attributed to the symmetric stretching vibration of the C–O–C link (Sengupta *et al*., 2015). This linkage indicates that *B. vulgaris* peels and pomace are polysaccharides hence contain sugars and pectins (Talari *et al.*, 2017). On the other hand, the band at 986.22 cm^{-1} is responsible to the bond deformation of the C–H bond, that is aromatic C-H in-plane bend and finally the band at 918.87 cm^{-1}

corresponds to the stretching vibrations of the C–COOH bond (de Assis, Margem, Loiola, & Monteiro, 2014).

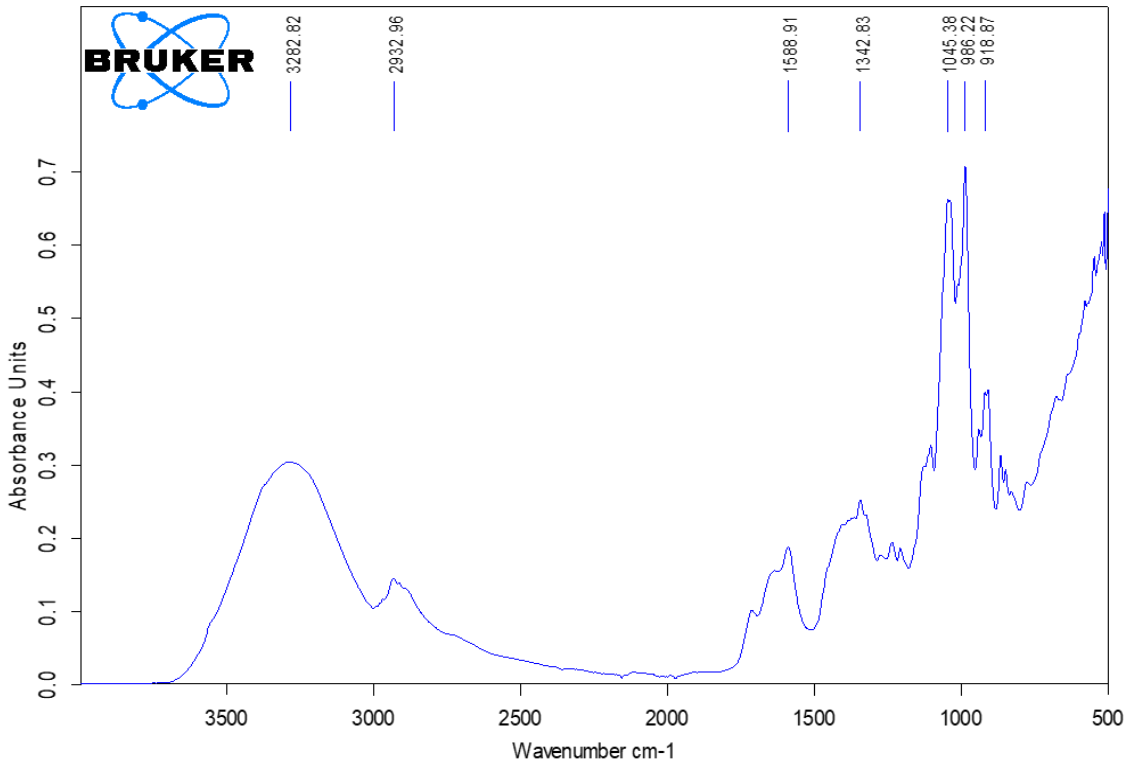


Figure 4.5. FT-IR spectrum of *B. vulgaris* peels and pomace methanolic extracts.

The colour of *B. vulgaris* peels and pomace may result from the vibrational properties of its molecules. Depending on pigment concentration, different colour tones and hues may emerge because of different structural conformations and different vibrational characteristics. Therefore, molecules exhibit constant movement because of the vibrations of the covalent bond network that occurs at different intensities guided by interchanging such as synchronous and asynchronous bond stretching, angle bending and dihedral torsions. Molecules vibrate at frequencies in the infrared (IR) spectral region, while the visible absorption spectra of molecules (for colour) are derived from the energy states of their constitutive electrons due to electric properties (Ai *et al.*, 2011; Cerezo,

Zúñiga, Requena, Ávila Ferrer, & Santoro, 2013). The vibrational and electronic properties of molecules influence each other through vibronic coupling. Thus, in pigments that appear with different structural conformations, these is associated to variation in the frequency, intensity or mode of molecular vibration (Galván, Solano, & Wakamatsu, 2013). There is evidence that vibronic coupling predicts the visible absorption spectra of biomolecules, including those of many biological pigments like betanins, flavins, carotenoids and anthraquinones (Ai *et al.*, 2011; Cerezo *et al.*, 2013; Jacquemin, Brémond, Ciofini, & Adamo, 2012). Vibronic coupling predicts the colours produced by pigments such as betanins (Jacquemin *et al*., 2012).

4.4 HPLC and LC-MS Analysis of Pigments

HPLC-UV and LC-MS is an invaluable means of separating and analyzing organic compounds such as pigments in plant extracts. Tentative identification of betalains can be deduced from their chromatographic behavior, and corroborative data may be provided by analysis of their absorption spectra via chromatograms.

4.4.1 Method Validation/Quality Control

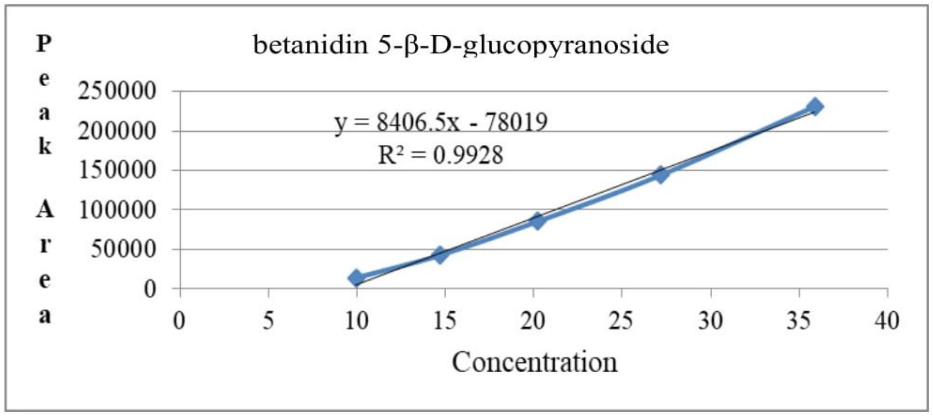
4.4.1.1 Calibration Curve Linearity and Range

Five different concentrations of standard betanidin 5-β-D-glucopyranoside solution ranging from from 10 to 35 ppm were analyzed using HPLC. The betanin standard concentration and respective responses (peak areas) from the HPLC-UV chromatograms were used to generate calibration graph regressed as x and y-axis respectively (Figure 4.6**a**). The results obtained showed a linear relationship. An average of three determinations in each standard concentration were applied. The calibration graph

showed a strong positive correlation between the concentration of the betanin standard and the signal.

The LC-MS calibration curve obtained by weighted (1/concentration) least-squares regression analysis of values ranging from 0.02 ng/mL to 50 ng/mL of betanidin 5-β-D-glucopyranoside standard. Figure 4.6**b** shows the reliability of betanin qualification in *B. vulgaris* peels and pomace extracts analyzed with TSQ LC-MS, exemplifying the robustness of this method in routine qualitative and quantitative determinations.

a



b

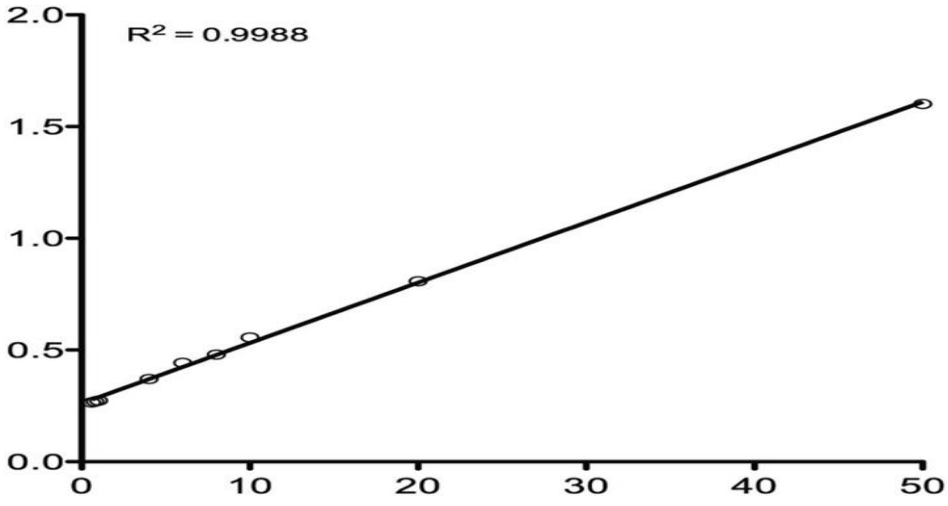


Figure 4.6. (a) Preparative HPLC calibration curve for betanidin 5-β-D-glucopyranoside standard; (b) Passing and Bablok fit of betanidin 5-β-D-glucopyranoside in *B. vulgaris* peels and pomace extracts determined with TSQ-MS.

4.4.1.2 **Repeatability**

The repeatability of the HPLC method was investigated by performing five repeated analysis of the betanin standard solution at concentrations ranging 10-35 ppm. This was based on the same day (for intra-day repeatability) and a different day (Inter day repeatability). The results showed that the average retention times were close to the retention time of betanin standard (betanidin 5-β-D-glucopyranoside) as seen in Table 4.8 and thus the % RSD for retention time and area provided satisfactory precision enabling continuation of analysis.

Table 4.8. Intra-day and Inter-day repeatability at various concentrations.

	Intra-day repeatability			Inter-day repeatability		
#	Concentration of standard Sample (ppm)	Retention time (min)	Peak Area (uV)	Concentration of standard Sample (ppm)	Retention time (min)	Peak Area (uV)
1	10	7.685	12181	10	7.588	14029
2	15	7.680	19543	15	7.592	19682
3	20	7.602	37832	20	7.612	19756
4	27	7.692	37004	27	7.794	37098
5	35	7.689	37832	35	7.691	50082

4.4.1.3 **Sensitivity**

The instrumental software (Shimadzu Lab Solutions Version 5.60SP2) detected signal to noise ratio 3:1 for the standard concentration of analyte betanidin 5-β-D-glucopyranoside at 0.2 ppm, which was the least concentration the analyte could be detected. By applying Equation 3.5 and 3.6, LOQ was found to be 0.8 ppm and 3.68 ng/mL for HPLC and LC-MS respectively at which the instrumental software assessed the signal to noise ratio of 10:1 in each case, which was within the required limit in HPLC and LC-MS/MS analysis. In addition, 1.21 ng/mL LOD for LC-MS was achieved.

4.4.2 HPLC Chromatograms and Betanin Identification

The present study employed a two-way strategy in betanin identification. The first is using the HPLC-UV to establish the retention time of sample and then secondly, using the quadruple LC- MS techniques to further elucidate their structure (Kumorkiewicz, 2017). The HPLC chromatograms of betanin were monitored at 538 nm for betanins. Betanin profiles are often subject to change especially during extraction and purification process due to pigment degradation. All these compounds identified in the present analysis have previously been described by Nemzer *et. al*., 2011.

The spectra for solvent blanking and before running the standard and sample respectively are shown in Appendix 6**a** and **b** respectively. Chromatogram in Appendix 6**a** had a 'ghost' peak probably indicating there were remnants of contaminants from previous analysis. Chromatogram in Appendix 6**b** had more 'ghost' peak resulting from pre-sample analysis. The blank was methanol with 1% formic acid. This mixture was also used to purge the HPLC completely for an hour before starting to run the standard with a flow rate of 3mL/min. This was done to get rid of impurities emaneting from previous injections. The injection of blank (the diluent used to dissolve the standard) was followed by the betanin standard, then another blank before injecting sample. The first blank showed a shorter peak at retention time of 9.85 minutes but the peak in the second blank become more pronounced at the same time of retention. This meant there was no carryover. The compounds were well dissolved and not "sticky". *B. vulgaris* peels and pomace extracts are sticky (apolar) hence neccesary to do blanking in between standard and sample analysis to help clean the column.

Chromatograms in Appendix 7, 8 and 9 **(c** to **g)** of betanin standard at different concentrations clearly demonstrates an outstanding peak (peak 1). This peak was assigned to betanin in the standard, which is responsible for red colour in *B. vulgaris* peels and pomace (Kumorkiewicz, 2017). Similarly, peak 1 as seen Appendix 10 (**h, i** and **j**) could be clearly identified in the plant sample by comparing the retention time results with the betanin standard runs.

Four distinct peaks were observed on the chromatographic pattern of *B. vulgaris* peels and pomace pure sample. The four betacyanin peaks in the red beetroot extracts were eluted at close retention times of 7.699, 7.890, 10.065 and 10.008 minutes respectively (Table 4.8). Betanin peaks observed at an average retention time of 7.699 min with peak area at average of 242256 uV from the triplicate sample runs. This finding corresponded with the results from the betanin standard which gave a similar peaks at around 7.662 as seen in spectras **(c-g)** in Appendix 7-9. The second highest peak (peak 2) in the *B. vulgaris* pure sample as in chromatograms (**h, i** and **j**), Appendix 10 was identical to that of isobetanin while peak 3 and 4 were attributable to betanidin and isobetanidin respectively due to their longer retention times assigned according to their retention time, maximum absorption length and previous studies (Mikołajczyk-Bator & Pawlak, 2016). The results from this study confirmed the presence of betacyanins in *B. vulgaris* peels and pomace methanolic extract. The respective average area percentages of the betacyanins of the extract detected at 538 nm were 38.16 % for betanin, 29.62 % for isobetanin, 20.12 % for betanidin and 12.10 % for isobetanidin since area % is directly proportional to eventual concentration. Red beetroot is a good source of red pigments called betalains which is part of betacyanins group of compounds (Guldiken *et al*., 2016)

(Table 4.9 and 4.10). The results coincides with UV-Vis absorbance maxima of betalain of 535-538 nm obtained in UV-Vis analysis.

Table 4.9. Chromatographic peak tables of *B. vulgaris* peels and pomace extract.

Chromatogram (h)	Peak#	Ret. Time	Area	Height	Area %	Height %
	1	7.602	213249	20871	37.6006	36.1860
	2	7.891	169707	20316	29.9232	35.2237
	3	10.425	116542	12743	20.5490	22.0937
	4	10.311	67644	3747	11.9272	6.4965
	Total		567142	57677	100.0000	100.000
Chromatogram (i)	Peak#	Ret. Time	Area	Height	Area %	Height %
	1	7.797	251263	24886	38.4177	33.2301
	2	7.889	192832	24693	29.4837	32.9724
	3	9.896	130214	18316	19.9095	24.4572
	4	9.902	79721	6995	12.1892	9.3404
	Total		654030	74890	100.0000	100.000
Chromatogram (j)	Peak#	Ret. Time	Area	Height	Area %	Height %
	1	7.697	262256	20102	38.4503	32.5070
	2	7.891	200879	19970	29.4516	32.2935
	3	9.875	135762	15730	19.9046	25.4370
	4	9.811	83168	6037	12.1936	9.7624
	Total		682065	61839	100.0000	100.000
	Key : Peak 1-Betanin, peak 2-isobetanin, peak 3-betanidin, peak 4-isobetanidin					

Table 4.10. Summary peak table of the plant extract chromatograms.

Peak#	Av. Ret. Time	Av. Area	Av. Height	Av. Area %	Av. Height %
1	7.699	242256	21953	38.1562	33.9743
2	7.890	187806	21659.67	29.61948	33.4966
3	10.065	127506	15596.33	20.12101	23.9960
4	10.008	76844	5593	12.10331	8.5331
Total		634412	64802	100.000	100.0000
Key: Av. =average; uV =microvolts					

4.4.3 Quantity Estimation of Betanin Content in *B. vulgaris* Extract

The estimation of betanin content in the methanolic extract was quantified using the ESTD method of quantification. The amounts of betanin contained in the plant sample

were expressed in mg/g, expressed as the external standard equivalent (betanidin 5-β-D-glucopyranoside) from the calibration curve. The method is due to established direct correlation between peak areas (absorbance/concentration) of the betanin standard and the sample under study.

Table 4.11. Content estimates of betanin compound in *B. vulgaris* peels and pomace sample.

Run	Betanin Retention time(tR)	Betanin Peak area(uV)	Betanin content (mg/g)
1	7.602	213249	3.47 ± 0.22
2	7.797	251263	3.92 ± 0.19
3	7.697	262256	4.05 ± 0.12
Mean($\bar{x}$)	7.699	242256	3.81 ± 0.31
Results are presented as mean values of three replicates ± SD; Expressed as mg/g			

Table 4.12. Percent composition of betalains in the plant extract.

Peak	Compounds	% of contribution in total betalain content
1	Betanin	38.16
2	Isobetanin	29.62
3	Betanidin	20.12
4	Isobetanidin	12.10

Results from the three HPLC-UV runs (Table 4.11) of the pure sample fractions yielded 3.47 ± 0.22, 3.92 ± 0.19 and 4.05 ± 0.12 mg/g betanin respectively, which averaged 3.81 ± 0.31 mg/g. Converting to mg/100g of dry weight resulted in 381.00 ± 31 mg/100 g betanin of dry weights. The results obtained were in agreement with the results obtained from Kechi *et al.* (2013) and Prieto-Santiago, Cavia, Alonso-Torre, & Carrillo (2020**)** findings, who established that the total betanin content of red beet were between 250 to 850 mg/100 g. The betalain compounds percentage contribution in the total betalain content in *B. vulgaris* peels and pomace extract are presented in Table 4.12. Betanin and

may be isobetanin were the predominant betacyanins in red *B. vulgaris* peels and pomace in the present study. Both probably constituted 38.16 % and 29.62 % respectively of the total betalain composition in the plant extract. They were characterized by significantly higher relative peak areas compared to other compounds. Betanin and isobetanin displayed 242256 uV and 187806 uV peak areas respectively. There were high chances that betanidin and isobetanidin were among the bioactive compounds in the *B. vulgaris* pomace and peels sample. They constituted about 20.12 % and 12.10 % respectively, which translated to 127506 uV and 76844 uV peaks areas respectively. Slatnar *et al.* (2015) identified the presence of fifteen compounds among them betanidin and isobetanidin. The results confirmed that betanin is the main compound present in high quantities in the red beetroot juice giving it red colouring according to Kathiravan *et al.,* 2014. In addition, the results projects and reaffirms that red *B. vulgaris* constitutes the richest source of betanins (Esatbeyoglu *et al.,* 2015).

This method afforded the choice of obtaining an external betanin standard that was commercially available for the quantification of related betanin in the sample. The basis of its selection and identification was due to its similarity with the betanin present in the test sample based on their similar retention times. The preliminary experiments afforded a characteristic HPLC profile of betanin present in the *B. vulgaris* peels and pomace extract. This preliminary analysis suggests that the external color of *B. vulgaris* peel and pomace relies on the relative concentration of betanins (red pigments) with maximum absorbance at around 538 nm (Lee *et al.,* 2014). This method is suitable for the identification and verification of characteristic fingerprints of betanins present in several plant materials used in medical, textile and other industries. It is a cost-effective,

accurate, approach for quantification of betanins and other betacyanin compounds. Good repeatability enables viable quality assurance systems for identification and quantification of individual betacyanins in plant extracts. Just like any other analytical method, minor modifications might be applied to fit into the changes in sample matrix and requirements of product specifications (Nemzer *et al.,* 2011).

4.4.4 LC-MS Analysis

Appendix 11(**a**, **b** and **c**) shows the chromatographic pattern of the pure *B. vulgaris* peel and pomace sample, betanin standard and mass spectrum of the sample respectively in LC-MS. The major components that could be present in the purified *B. vulgaris* peel and pomace sample were identified to be betanin (*t*R = 7.71 min, $[M+H]^+$ = 551.1097) and isobetanin (*t*R = 8.81 min, $[M+H]^+$ = 551.1097). The compounds were identified by means of a comparison of their retention time, MS spectra, previous data (Lee *et al.,* 2014; Nemzer *et al.,* 2011) and through interpretation of the obtained fragmentation spectrum. The *t*R of betanin standard (betanidin 5-β-D-glucopyranoside) was observed to be 8.189 minutes. The MS data of red beetroot betalains are presented in Table 4.11 showing the percent contribution to total betalain composition, retention times, m/z values and fragments of respective betalains.

Thirteen compounds were identified among them eleven betalain compounds (Appendix 11**a**) and Table 4.13. MS analysis of peak 2 revealed the pseudomolecular ion for betanin with mass charge (m/z) of 551.1 $[M + H]^+$. The mass spectrum (Appendix 11**c**) shows the parent ion at m/z of 551.1497. The subsequent fragmentation of the parent ion yielded daughter ions assigned to betanin (betanidin 5-glucoside) and isobetanin both with similar m/z of (551.1 $M + H]^+$) but different *t*R of 7.71 minutes and 8.81 minutes

respectively. The m/z 387.1 $[M + H]^+$ may be corresponding to betanidin and was produced probably after further fragmentation of the pseudomolecular ions (betanin at peak 2 and isobetanin at peak 4) of m/z 551.1 $[M + H]^+$ taking into account the previous data (Nemzer *et al.,* 2011). The fragment ion at the m/z of 389.0966 indicated that this ion could have been obtained by glucose loss. Peak 1 and 3 had identical MS spectrum with pseudomolecular ion of m/z 389.1 $[M + H]^+$ but had different retention times. Peaks 1 and 3 might have been having similar epimers causing a different retention time and possibly corresponded to 2-*O*-glucosyl-betanin and 2-*O*-glucosyl-isobetanin respectively. Compound at peak 4 that yielded the pseudomolecular ion of m/z 551.1 $[M + H]^+$ was identified as isobetanin while peak 5 was identical to betanidin. *B. vulgaris* peels and pomace extract was also pointed to have small quantities of ferulic acid hexoside, identified as compound at peak 6 with m/z of 355.1$[M + H]^-$. This confirms the occurrence of free cyclodopa glucosides in red beetroot, as ferulic acid hexosides compounds, which are glucosides. Ferulic acid hexosides play a role as intermediate of betanin biosynthesis (Lee *et al*., 2014; Slatnar *et al.,* 2015). Peaks 7 and 8 revealed pseudomolecular ions of m/z 431.1 $[M + H]^+$ and 345.1$[M + H]^+$ respectively and were suspected to be 17-Decarboxy-neobetanin and isobetanidin (17-Decarboxy-betanidin) respectively. The two compounds are part of degradation products of betanin, including isobetanin at peak 4 (Lee *et al*., 2014; Slatnar *et al*., 2015).

Other degradation products of betanin are; 17-Decarboxy-betanin; 2, 17-bidecarboxy-betanin and 2, 17-Bidecarboxy-neobetanin that were identified as peaks 10, 11 and 12 respectively with m/z of 507.2 $[M+H]^+$, 475.1 $[M-H]^-$ and 461.2 $[M+H]^+$ respectively (Nemzer *et al.,* 2011).

Table 4.13. Betalains identified in *B. vulgaris* peels and pomace extract.

Compound no.	Compound name	Rt [min]	[m/z]	Ions
1.	2-*O*-glucosyl-betanin	6.97	389.1	$[M+H]^+$
2.	Betanin	7.71	551.1	$[M+H]^+$
3.	2-*O*-glucosyl-isobetanin	7.98	389.1	$[M+H]^+$
4.	Isobetanin/isobetanidin 5-glucoside	8.81	551.1	$[M+H]^+$
5	betanidin	9.46	387.1	$[M+H]^+$
6.	Ferulic acid hexoside (4-glucoside)	9.68	355.1	[M-H]-
7.	17 -Decarboxy-neobetanin	10.93	431.1	$[M+H]^+$
8.	Isobetanidin/17 –Decarboxy-betanidin	11.67	345.1	$[M+H]^+$
9.	Neobetanin	12.13	549.1	$[M+H]^+$
10.	17 -Decarboxy-betanin	13.72	507.2	$[M+H]^+$
11.	2,17-bidecarboxy-betanin/isobetanin	14.18	475.1	[M-H]-
12.	2,17-Bidecarboxy -neobetanin	14.78	461.2	$[M+H]^+$
13.	Isovitexin; apigenin 6-C- glucoside	14.86	433.2	$[M+H]^+$

Neobetanin that was identified in peak 9 with m/z of 549.1 $[M + H]^+$ is known to be among the artefacts formed during isolation of betanin in production process (Slatnar *et al.,* 2015). Peak 13 gave the molecular ion of m/z 433.2 $[M + H]^+$ that resembled isovitexin (apigenin 6-C- glucoside). Isovitexin is a flavanol confirming the presesence of flavanols as bioactive compounds in *B. vulgaris* peels and pomace sample (Nemzer *et al.*, 2011; Slatnar *et al.,* 2015).

4.5 Optimization and Statistical Modelling of Cotton Fabrics Dyeing

The dyed and mordanted fabrics after each experimental run were subjected to SP60 X-Rite Spectrophotometer to deduce their relative change in colour strength (%). The respective relative colour strengths percentages as in Table 4.14 were positioned as *y* (response) and regressed with the independent variables which affect dyeing; temp.(°C) denoted A; time (mins.) denoted B and pH denoted C. Equation 3.2 was fitted to form a regression equation as in Equation 4.2 below;

$$y = 5.59 + 0.0013\,A + 0.0239\,B - 0.033\,C \qquad \textit{Equation (4.2)}$$

The regressed variables (temp., A; time, B and pH, C) by way of ANOVA are demonstrated in Table 4.15. The p-values of the variables were greater than the common α level of 0.05, which indicated that they were not statistically significant. Other factors could have also affected dyeing and relative change in colour strength such as dye concentration, mordanting, etc. Therefore, use of mordants to fix colour onto fabrics and modify colour was required according to Kulkarni *et al.,* 2011, and Konar & Samantha, 2011. The adjusted R^2 values were well within the acceptable limits indicating a good fit model.

The response surface plot represented in Figure 4.11a, b and c reveal some relations between the response and the variables. The investigated variables have some effect on dyeing in terms of linear, quadratic and cross terms leading to optimized dyeing conditions in Table 4.16. Increase in dye bath concentration leads to more dye transfer to the fabric, hence a higher apparent depth of colour (Rather *et al.,* 2016b).

Table 4.14. Experiments planned in CCD for dyeing optimization and response (y).

Run Order	Temp.	Time, mins	pH	Response(y) (Relative Colour Strength %)
1.	55	75	6.5	11.0
2.	-2.155	75	6.5	5.1
3.	112.155	75	6.5	7.1
4.	55	148.485	6.5	5.4
5.	55	1.515	6.5	3.9
6.	55	75	10.5825	8.2
7.	55	75	2.4175	10.3
8.	90	30	4	6.7
9.	20	30	9	6.0
10.	90	120	9	7.1
11.	55	75	6.5	9.5
12.	90	120	4	8.9
14.	20	120	9	7.6
15.	90	30	9	7.2
16.	20	120	4	5.3
17.	20	30	4	7.0

Table 4.15. ANOVA for dyeing process.

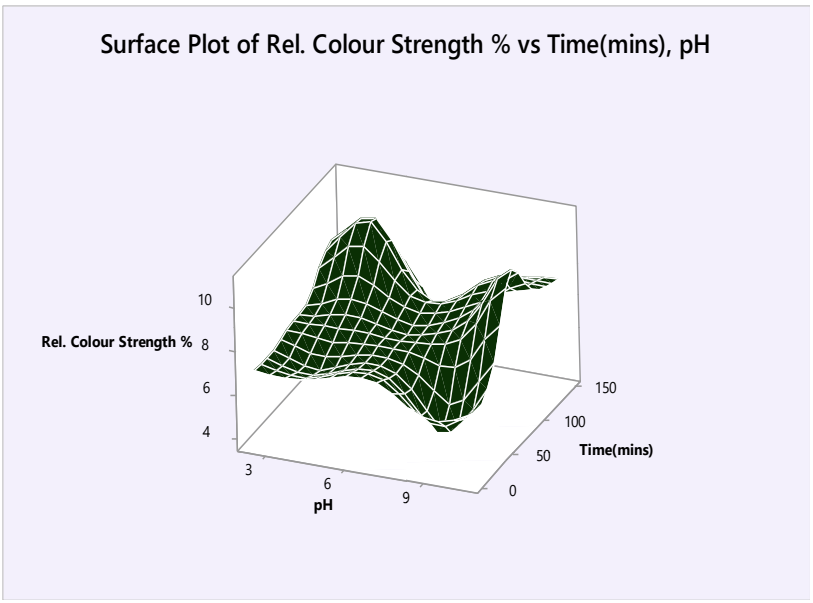
Properties	df		SS	MS	F	Significance F	P-value
Intercept	3		5.74209	1.9140	0.4833	0.69962	0.01026
Temp.,^{0}C	13		51.4885	3.9607			0.60370
Time,hrs	16		57.2306				0.29970
pH							0.99684
Regression Statistics							
Multiple R		0.316753					
R^2		0.100333					
Adjusted R^2		-0.10728					
Standard Error		1.990139					

Table 4.16 Optimized dyeing conditions.

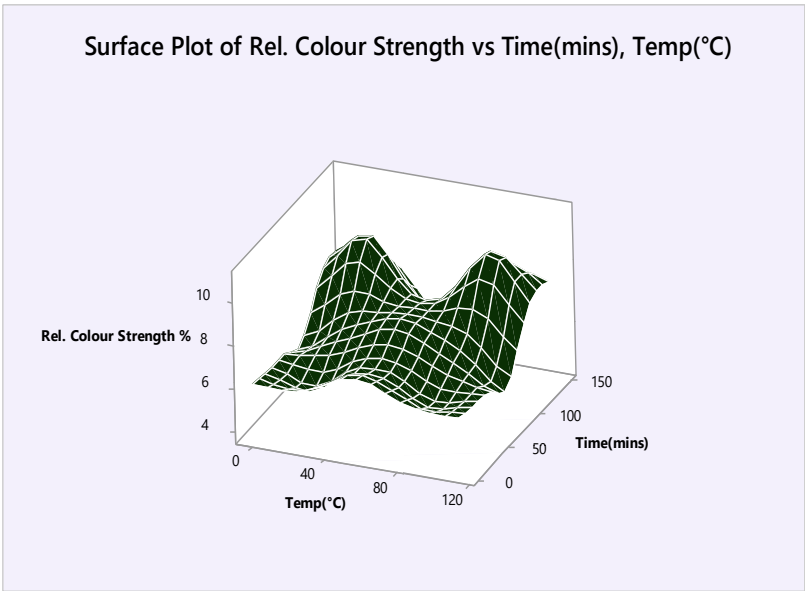
Parameter	Optimal Values
Temperature (^{0}C)	55
Time (minutes)	75
pH	6.5

The significance of the quadratic terms in Equation 4.2 indicates that the dyeing variables (dyeing temperature, pH and time) have impact on dyeing efficiency, even though their effects on the relative colour strength percent were non-significant. Figure 4.7**a**, **b** and **c** shows the interaction effects of time, pH and temperature on colour compounds. When temperature is maintained at constant level as in Figure 4. 7**a,** the increase in time and decrease in pH leads to increased dye absorption. The highest dyeing efficiency of cotton fabrics cannot be seen at low time and high pH. In Figure 4. 7 **b**, when pH is maintained as a constant, slight increase in time (50-100 mins) leads to more percent dye intake but too much temperature (>80 °C) can denature the colour compounds, such as betalains leading to low dye uptake by cotton fabrics resulting in poor dyeing. Many phytochemicals are denatured by high operation time and temperature as reported by Ravichandran *et al*. (2013). Moderate temperature favours stability of betanin pigment hence unlimited absorption onto cotton fabrics. This correlation can be explained by the findings of Gokhale & Lele (2014) who investigated the impact of hot air on *B. vulgaris* peels and pomace colour compounds. In Figure 4.7 **c,** when time remains constant, moderate temperature (40-80 °C) and low pH (4-7) favours optimum dyeing. At very high pH and high temperature, betatains are destroyed and chromphoric effect is lost (Ravichandran *et al.*, 2013).

a


Surface Plot of Rel. Colour Strength % vs Time(mins), pH
Rel. Colour Strength %
10
8
6
4
3
6
9
pH
0
50
100
150
Time(mins)

b


Surface Plot of Rel. Colour Strength vs Time(mins), Temp(°C)
Rel. Colour Strength %
10
8
6
4
0
40
80
120
Temp(°C)
0
50
100
150
Time(mins)

c

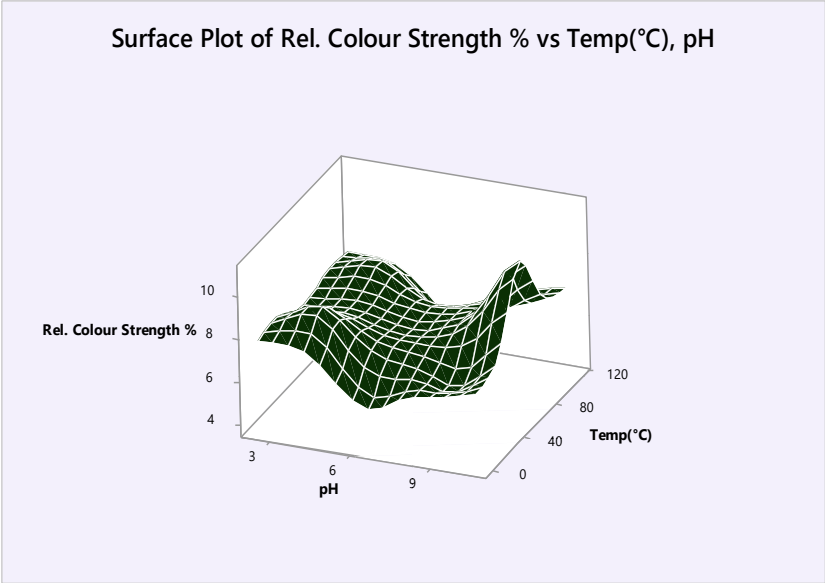


Figure 4.7. RSM for dyeing of cotton substrate using *B. vulgaris* peels and pomace extract: a) Correlational effect of time and pH on relative colour strength percent; (**b)** Correlational effect of time and temperature on relative colour strength percent; (**c)** Correlational effect of temperature and pH on relative colour strength percent.

4.5.1 Effect of Mordants on K/S and CIELab Coordinates

Natural dyes need metal ion for adsoption of dye by forming an insoluble composition precipitate on the surface of the fibres and this give the fabric a range of bright colours (Tera, Elnagar, & Mohamed, 2012); Uddin, 2015). Mordants fix and modify colour onto fabrics as well as enhancing the colour fastness of dyed fabrics (Kulkarni *et al.*, 2011; Konar & Samantha, 2011). *B. vulgaris* peels and pomace dye is water soluble and contains carbonyl and hydroxyl groups, hence it can interact with cotton fabric via hydrogen bonding (Sarhan & Salem, 2018). Natural mordants of the order tannic acid-alum gave the best shades with post mordanting. Aluminum ions have a strong affinity for cellulose and readily serve as a bridge between multiple dye molecules and/or between the fiber and dye. The relative change in colour strength of the dyed cotton is dependent on the concentration of mordants (Table 4.17). Reported concentrations of mordants have ranged from 2% to 100% o.w.f. It was observed that the relative colour

strength increases gradually with a dyeing time of up to 75 minutes and decreased when the time increased further. Similarly, relative colour strength increased with increase in mordant concentration. At higher mordant concentration there is little leaching of color from dyed cotton samples. The results of present study correlates well with previously reported work by Ali, Islam, & Mohammad (2016).

In pre-mordanting with natural mordants (Table 4.17), it is suspected to have an initial increase in equilibrium exhaustion from 10% to an optimal concentration of 30% o.w.f, beyond which the pecentage dye uptake reduced. This reduction is attributed to excess concentration of mordants leading to the aggregation of the extract molecules causing a reduction in extract solubility (Ali *et al.,* 2016). This enhanced precipitation led to difficulty in penetration of *B. vulgaris* peels and pomace dye molecules into the fabric during dyeing. The simultaneous mordanted dyed cellulosic fabrics had lower dye-uptake than the pre-mordanted dyed fabrics. This was attributed to mordant/dye interaction that reduced quantity of dye molecules diffused in to be absorbed by the cotton matrix. These observations were similar with findings made by Yusuf *et al*. (2016) that biomordants such as *Acacia catecheu* and tannic acid improves color and fastness properties. In post-mordanting, it can be seen from Table 4.17 and 4.18 that post-mordanted dyed fabrics have higher dye-uptake than other mordanted dyed fabric. This was due to enhancement in affinity of dye for the cellulosic fabric by mordant used after dyeing the fabric (Uddin, 2015).

Table 4.17. Effect of mordant concentration on relative % colour strength.

Mordant	Method	Relative Colour strength % At 20-50%			
		Mordant Concentration			
		20%	30%	40%	50%
$CuSO_4$	Pre mordanting	4.1	5.0	5.1	5.8
	Simultaneous mordanting	4.3	5.1	7.4	7.7
	Post mordanting	4.0	5.4	7.3	7.7
$FeSO_4$	Pre mordanting	4.5	4.7	6.3	6.8
	Simultaneous mordanting	5.5	5.7	5.6	7.1
	Post mordanting	6.2	6.3	6.7	9.1
$K_2Cr_2O_4$	Pre mordanting	4.1	4.6	4.4	4.8
	Simultaneous mordanting	4.2	4.5	4.9	5.3
	Post mordanting	3.6	3.9	4.3	4.1
Tannic acid-alum	Pre mordanting	3.9	5.3	6.0	7.6
	Simultaneous mordanting	5.1	6.7	7.0	10.3
	Post mordanting	5.4	6.0	7.0	11.0

In Table 4.18, the cotton fabric showed higher L* value (80.34) in dyed but non-mordanted fabric, gradually decreasing towards mordanted fabrics. The order of tannic acid-alum combination under post-mordanting had the lowest L* value (69.41) indicating change in colour to darker due to absorbed *B. vulgaris* peels and pomace dye enhanced by the mordant resulting in high colour saturation as depicted by its chroma (C of 15.09). The tone of samples were much towards yellow/orange and red as indicated by values of a* and b* which mostly positive indicating shades were moving towards red and yellowness/orange. In general, when the L* values are high, a* values becomes low values meaning shades become deeper. This was attributed to concentration gradient of dye on fiber via adsorption (Ali *et al.,* 2016, Rather *et al.,* 2016). These observations were in agreement with Sufian, Hannan, Rana, & Huq (2016). These changes could be attributed to resonating structures of dyes. The natural mordant probably limited metal – dye complexion which encouraged dye absorption. Copper (II) sulphate mordant and

potassium dichromate had some negative a* values meaning the shades were aligning to yellowish/orange than red in appearance.

The values of b* (yellowness-greenness) were greater than those of a* (yellowness-redness) in dyed fabrics using *B. vulgaris* peels and pomace extracts, meaning that the shades were more yellowish or lighter than red in appearance. The dye could have degraded to lighter shade. Post mordanting method showed the best result as depicted by its chroma (C*) and relative change in colour strength percentages, especially by using the order of tannic acid-alum combination as well as iron (II) sulphate having relative colour strength percentages of 11.0 % and 9.1 % respectively as seen in Table 4.18. The high value of chroma in alum-tannic acid is reflected in its high brilliance appearance. The dyed but non-mordanted fabric exhibited the lowest degree of saturation with a low value of C (1.36). Dye molecules from solution diffuse into the fabric matrix, followed by adsorption of dye molecules at surface of the fabric. The adsorbed dye molecules are then absorbed into the fabric matrix, followed by chemical fixation of dye molecules unto the mordanted fabric (Bukhari *et al*., 2017).

In Table 4.19, colour absorption values (K/S) for the *B. vulgaris* peels and pomace dye extracts increased gradually as wavelength increased from the violet to the red end (400 to 700 nm) of the visible spectrum (Figure 4.8). Reflectance resulted in characteristic mixture of yellowish to light reddish colours of dyed fabrics. Light reflected from *B. vulgaris* peels and pomace dye in the fabrics had more than 50% spectral colours occupying the yellow to red wavelength bands as observed in the reflectance values between 580 to 700 nm. The strong coordination tendency resulting in enhanced interaction between the tannic acid-alum mordants and *B. vulgaris* peels and pomace dye

extract could have been responsible for the high dye uptake between 530 and 700 nm as represented by curve of the order of tannic acid-alum combination in Figure 4.8 (Rather *et al.,* 2016a, 2016b). The color strengths (K/S) recorded for post-mordanting method were; (3.60), (3.92), (1.20) and (4.24) for copper sulphate, ferrous sulphate, potassium dichromate and tannic acid-alum respectively. The post mordanting method gave the best color strength measured in all the mordants in the present study while the simultaneous mordanting method gave the second best color strength according to the color strength data in Table 4.17. However, the order of tannic acid-alum combination gave the greatest color strength of (4.24) among the mordants implying that the dye extract in the current study has demonstrated to be stable in pH below 6, confirming Sturzoiu *et al.* (2011) findings that more betanin and colour stability was observed from beets at low pH conditions during extraction. These results forecasted that the natural dye has the capability to impart colour on cotton fabrics with the help of mordants, hence competing with synthetic dyes such as R. Orange HER. In the control test, dyed fabric without the application of mordants had low color strength (0.11) indicating that some colour was absorbed but not enhanced (Table 4.19). Reflectance values were obtained from the spectrophotometer from which the K/S values were calculated using the Equation 3.7. Additionally, the spectrophotometer deduced the coordinate values (L*, a* and b*) that were then used to calculate chroma (C), hue angle (h°) and total colour change (ΔE**)** by applying Equation (3.8, (3.9) and (3.10) respectively.

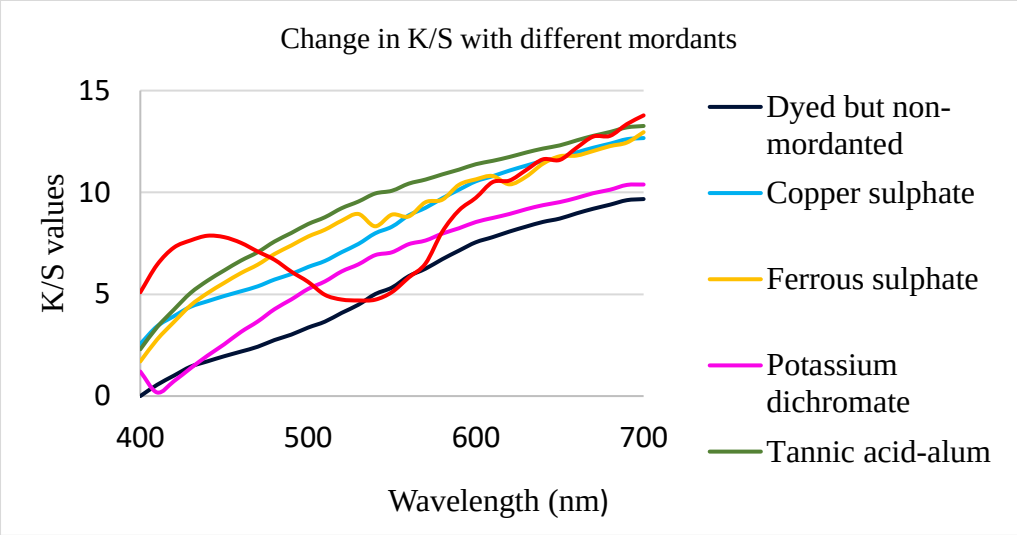


Figure 4.8. Spectral reflectance curves of cotton fabrics dyed with *B. vulgaris* peels and pomace extract enhanced by different mordants and methods in comparison with R. Orange HER dyed cotton fabric.

Table 4.18. Average colour coordinates of specimen fabrics.

Mordant/Dye	Method	CIELab cordinates						
		L*	a*	b*	C*	h°	Δ E	Relative Colour strength %
Dyed, no Mordant		80.34	-0.10	1.36	1.36	-85.79	41.29	3.4
$CuSO_4$	Pre mordanting	77.56	-0.19	8.52	8.52	-88.72	35.62	5.1
	S.mordanting	75.62	-0.03	10.04	10.04	-89.83	33.26	7.7
	Post mordanting	73.52	0.44	11.84	11.85	87.87	30.59	7.4
$FeSO_4$	Pre mordanting	76.70	1.94	8.85	9.06	77.63	34.35	5.5
	S. mordanting	75.60	0.42	9.48	9.49	87.46	33.36	6.7
	Post mordanting	71.13	0.50	11.88	11.89	87.59	28.41	9.1
$K_2Cr_2O_4$	Pre mordanting	78.86	-0.57	7.07	7.09	-85.39	37.44	4.2
	S. mordanting	77.88	0.14	7.03	7.03	88.86	36.46	4.8
	Post mordanting	75.86	0.50	7.11	7.13	85.98	34.57	5.3
Tannic acid-alum	Pre mordanting	77.09	2.74	9.34	9.73	73.65	34.40	5.3
	S. mordanting	73.91	5.48	7.53	9.31	53.95	32.07	7.0
	Post mordanting	69.41	9.47	11.75	15.09	51.12	26.25	11.0
R. Orange HER	Exhaust dyeing	66.47	22.01	-5.32	22.64	-13.55	37.94	11.3

S = Simultaneous

The tone of samples were much towards yellow and red as indicated by values of a* and b* which were mostly positive indicating shades were moving towards red and yellow/orange. In general, the high L* and low a* values means brighter shades. This is attributed to concentration gradient of dye on fiber via adsorption (Ali *et al*., 2016, Rather *et al*., 2016). These observations were in agreement with Sufian *et al*. (2016). The natural

mordant probably limited metal–dye complexion which encouraged dye absorption. Copper (II) sulphate mordant and potassium dichromate had negative a* values meaning they shifted the colour towards greenish.

Table 4.19. Reflectance and K/S values of dyed-mordanted cotton fabric.

Mordant/Dye	Method	R (%) at λ 400nm	K/S
Dyed , Not Mordanted		1.58	0.11
$CuSO_4$	Pre mordanting	6.98	2.56
	Si. mordanting	8.74	3.42
	Post mordanting	9.16	3.60
$FeSO_4$	Pre mordanting	5.21	1.70
	Si. mordanting	7.42	2.80
	Post mordanting	9.74	3.92
$K_2Cr_2O_4$	Pre mordanting	3.14	0.73
	Si. mordanting	1.80	0.18
	Post mordanting	0.24	1.20
Tannic acid-alum	Pre mordanting	6.44	2.30
	Si. mordanting	8.65	3.38
	Post mordanting	10.39	4.24
R. Orange HER	Exhaust dyeing	12.11	5.10

R=Reflectance, Si = Simultaneous,K= absorption coefficient, S = scattering coefficient

4.5.2 Effect of Mordants on Percent Absorption of Dye

From the optical densities before and after dyeing, the overall mean of percent absorption of dye by cotton fabric in combination with mordants are illustrated in Table 4.20. Copper (II) sulphate and the order of tannic acid-alum combination recorded highest values in simultaneous and post-mordanting methods while iron (II) sulphate recorded highest values in pre-mordanting method. Mordanted samples recorded higher values than unmordanted samples. Basing on relative colour strength percentages (Figure 4.9), post mordanting method showed the highest uptake of the dye (Figure 4.10). Cotton fabric dyeing with the combination of mordants showed some significant dye absorption.

Even though cotton has weak and low ability to form coordination complexes with dye molecules, mordanting improved its dye uptake capability (Tiwari *et al.*, 2011).

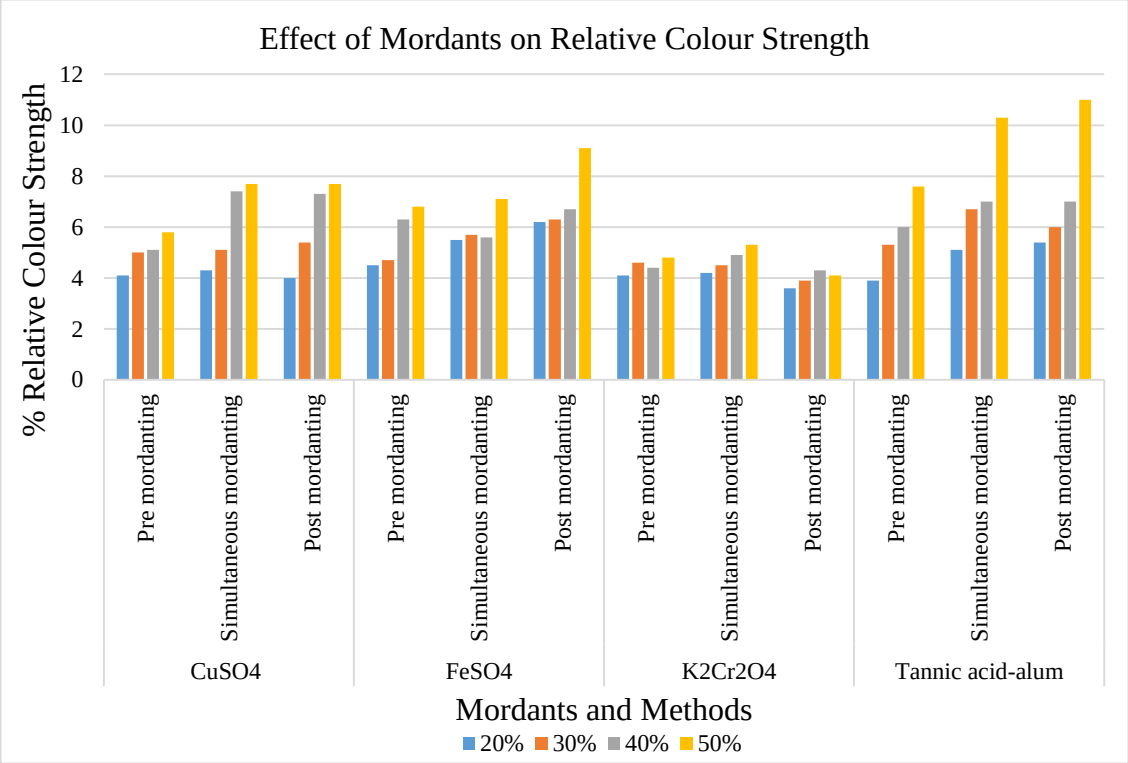


Figure 4.9. Effect of mordant concentration and methods on relative colour strength.

Table 4.20. Percent absorption of the cotton dyed fabrics.

Mordant		Pre mordanting	Simultaneous mordanting	Post mordanting
Without mordant	12±1.02	-	-	-
Copper (II) sulphate		13.68 ± 0.12	13.71 ± 1.21	14.11 ± 1.10
Iron (II) sulphate		13.97 ± 0.23	13.48 ± 0.62	13.87 ± 0.94
P. dichromate		12.82 ± 0.26	12.90 ± 0.88	13.08 ± 0.77
Tannic acid-alum		13.31 ± 0.70	13.96 ± 0.42	14.17 ± 1.66
Results presented as mean values of three replicates ± SD				

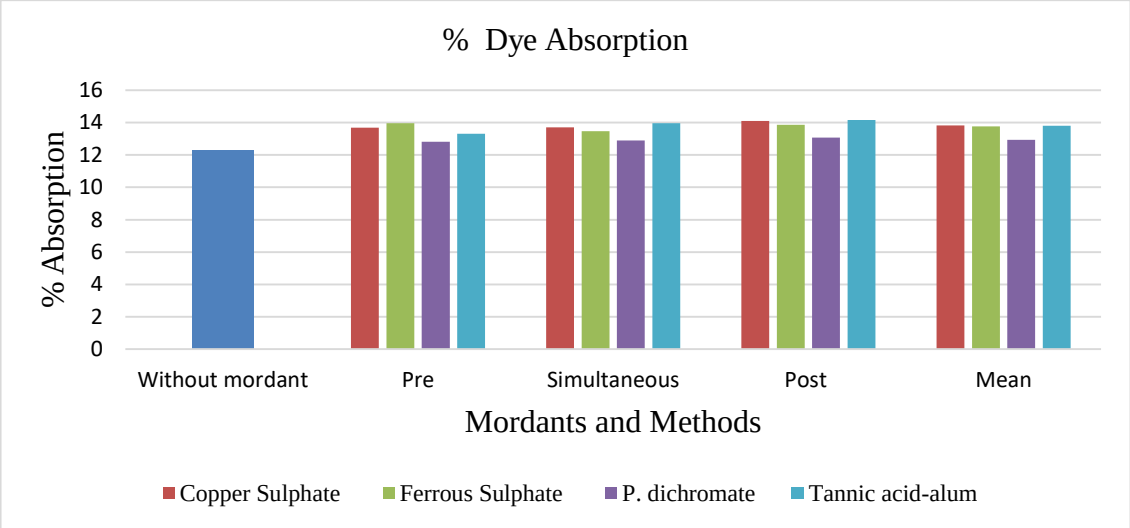


Figure 4.10. Percent absorption of the *B. vulgaris* peels and pomace dye in combination with metallic and natural mordants employing pre-mordanting, simultaneous mordanting and post-mordanting methods.

4.6 Colour Strength Equivalence, Ceq

The color strength equivalence (Ceq) and the color mass equivalence (Meq) of the *B. vulgaris* peels and pomace dye to commercial Reactive orange HER was determined from the Equation 3.11 taking into consideration the dilution factor of the extract (Aextr) as applied by Kechi *et al*. (2013) when they compared colour strength of Ethiopian dye plants with synthetic dye (Reactive Red 4).

The theoretical Ceq and Meq for the *B. vulgaris* natural dye extract at the highest absorbance (Aextr) were calculated as equivalent to commercial Reactive Orange HER dye and tabulated as shown in Table 4.21.

Table 4.21. Colour strength and mass equivalence; natural dye vs R. orange HER.

Absorbance(Aextr)	Dilution factor(DF)	Ceq,gdm^{-3}	Meq,g kg^{-1}
1.33	10	1.68 ± 0.21	33.6 ± 4.2
1.28	10	1.61 ± 0.38	32.2 ± 7.6
1.30	10	1.64 ± 0.52	32.8 ± 10.4
1.31	10	1.65 ± 0.43	33.0 ± 8.6
1.29	10	1.62 ± 0.22	32.4 ± 4.4
Mean($\bar{x}$)		1.64 ± 0.35	32.8 ± 7.04
Results presented as mean values of three replicates ± SD			

The Ceq values obtained averaged at 1.64 ± 0.35 gdm^{-3} while the corresponding Meq values averaged at 32.8 ± 7.04 at g kg^{-1} . The results indicated that 1 kg *B. vulgaris* peels and pomace dye could yield approximately 32.8 ± 7.04 g kg^{-1} on average equivalent of commercial R. Orange HER. The results corroborates to the method and findings of Kechi *et al*. (2013) who had used the similar method, and reported absorbance ranging from 0.3 to 28.1 for dyes extracted from Ethiopian dye plants whose Ceq and Meq were 0.62-2.9 g dm^{-3} and 0.62-57.9 g kg^{-1} respectively to commercial dye (R. Red 4). This demonstrates that *B. vulgaris* peels and pomace dye has immense potential for application as a source of natural dyes on cotton substrates. Color strength equivalence is a measure of the intensity of the color that depends on the nature of chromophore and the auxochromes present in the dye molecule. The nature of chromospheres and auxochromes varies in different crude natural dyes from different dye plant sources. These explain the existing relationship between color strength equivalence and dye yield of plant sources (Kechi *et al.*, 2013; Prieto-Santiago et al., 2020**)**.

4.7 Colour Fastness Properties Tests

Cotton can be dyed without mordants by using *B. vulgaris* peels and pomace extract but we do not expect excellent shades. To obtain excellent shades we require mordants. In this experimental study, different techniques of mordanting change the shades or enhances colour. The wash fastness results of all samples were found to be in the range of moderate to fairly good ratings of 3–4/5 in all the mordants. In Table 4.22, colourfastness to laundering in pre-mordanting was fair especially for $CuSO_4$ and $K_2Cr_2O_4$ with values of 3/4 and 3 respectively. However, post-mordanting method in tannic acid-alum and $FeSO_4$ positively affected the color change with no staining in wash fastness results ranging 4-4/5, which is very good to excellent. Reported color change ratings in laundering of mordant natural dyes on cotton, ratings varied depending on mordant (Francine, Jeannette, & Pierre, 2015), with ratings of 4-5 (slight-to-no change) to 2 (visible change) on the Color Change Gray Scale. Staining ratings also ranged from 4-5 to 2 (Francine *et al.*, 2015). The highest staining of cotton fabrics is because the unfixed dyes or surface excess effect causes a lot of staining. According to Wangatia, et al. (2015) the greatest retention of colour in the fabric after washing was obtained when appropriate mordant was used on the dyed fabrics.

Colourfastness with respect to dry rubbing was very good for all the samples with results within 4-5. Dry rubbing post mordanted with tannic acid-alum had excellent outstanding results of 5. It was found that assessment result for colour change and staining due tro rubbing was very good in simultaneous mordanting technique for $CuSO_4$ and $FeSO_4$ mordants in all the fastness tests with values ranging from 4-5.

Table 4.22 shows that all the mordants produced observable change in color fastness properties in comparison to that without a mordant. The lowest non-mordanted fabric being 2/3 value while highest mordanted had 5. This was true for all mordanting technique except; pre-mordanting of $CuSO_4$, $FeSO_4$ and $K_2Cr_2O_4$ mordants in respect to their light fastness for colour change and staining, and acid perspiration colour change. In addition, tannic acid-alum mordants also exhibited no observable change at pre-mordanting in respect to light fastness for colour change and staining. The outlined findings were further consistent in simultaneous mordanting; $CuSO_4$ mordant exhibited no change at light fastness for colour change and staining; $K_2Cr_2O_4$ mordant displayed no observable difference at light fastness for colour change and staining, and acid perspiration while $FeSO_4$ exhibited similar results at light fastness for staining. Furthermore, the statement was true for post mordanting method for both $CuSO_4$ and $FeSO_4$ mordants at light fastness for colour change and acid perspiration for colour change, again consistent in $K_2Cr_2O_4$ mordants in respect to colour change and staining in light fastness. The mordants on the fabric surface led to complex interaction between *B. vulgaris* peels and pomace dye extract and metal ions that prevented the possible photochemical oxidation of the natural dye in sunlight.

The medium resistance to perspiration by all the three mordanting techniques was attributed to the fabric construction. This was a light fabric (low GSM fabric) with plain weave in that it allows enough time and space for alkali and acid to react with the fabric colour (Mishuk & Rahman, 2014).

Post mordanted cotton fabrics demonstrated better fastness results among wash, light, rubbing and perspiration fastness as seen in tannic acid-alum, $FeSO_4$ and $CuSO_4$ with

mean ratings of 4-5 in colour change and staining grayscale. Post-mordanting technique for tannic acid-alum posted the best results in both colour change and staining with values in the range of 4/5-5 meaning very good to outstanding. Besides, simultaneous mordanting of tannic acid-alum gave low rating in colour change and staining as compared to its post-mordanting. This was connected to neutrality of the solution because of the alkaline media. All these findings corroborates with those of Sufian *et al*., (2016) in comparative study of fastness properties of avitera reactive dyes on cotton knit fabric.

It was also established that assessment result for color change was very good for all samples as compared to colour staining that is a very advantageous property for dyeing of cotton fabric with natural dyes. In this case, the tannic acid-alum mordant can also displace metallic mordants which are pollutants since they can also fix or hold dye on the substrate. These findings were in agreement with Rather *et al.* (2016b) in dyeing of natural fibres (wool) using *Adhatoda vasica* natural dye and evaluation of colour fastness properties.

From the table, there was no significant staining observed on the adjacent undyed cotton fabrics. In general, the fastness properties of dyed cellulosic fabrics were modified upwards by mordanting.

Table 4.22. Colourfastness rating to colour change and staining.

Mordant	Method	Washing IS 105-C06:2010		Rubbing IS 105-D02:2000				Light IS 105-B02:2000		Perspiration IS 105-E04:2000			
				Dry		Wet		C	S	Acid		Alkaline	
		C	S	C	S	C	S			C	S	C	S
$CuSO_4$	Pre mordanting	3/4	3/4	4/5	4	3/4	3/4	4	4	4	4	4	4
	Simultaneous	4	4	5	4	4	4	4	4	4/5	4	4	4
	Post mordanting	4	4/5	5	4/5	3	4/5	4	4/5	4	4/5	4	4/5
$FeSO_4$	Pre mordanting	4	4/5	4	4/5	4	4	4	4	4	4/5	4	4/5
	Simultaneous	4/5	4	4/5	4	4	4	4/5	4	4/5	4	4	4
	Post mordanting	3/4	4/5	4	4/5	3/4	4	4	4/5	4	4/5	4	4/5
$K_2Cr_2O_4$	Pre mordanting	3	3/4	4	3/4	4	3/4	4	4	4	3/4	3/4	4
	Simultaneous	3/4	4	4	4	4	4	4	4	4	4	3/4	4
	Post mordanting	4	4	4	4	3/4	4	4	4	3/4	4	4	4
Tannic acid-alum	Pre mordanting	3/4	4	4	4	4	4	4	4	4/5	4	4	4
	Simultaneous	4	4	4	4/5	4	4	4/5	5	5	4/5	4	5
	Post mordanting	4/5	4	5	5	4	4	4/5	5	4/5	4/5	4	4
Without mordant		3	2/3	3	3/4	3	3/4	4	4	4	3/4	3	3/4

C = colour change, S = staining

The metallic salt mordants possess special properties of chelating dye molecules to form complex in dyed fabric through increased interaction between cotton functional groups and dye functional groups (hydroxyl and carbonyl groups). Thus the mordanted dyed fabrics have higher fastness properties than unmordanted fabric. This is directly attributed to the increase in relative color strength values of dyed and mordanted cotton samples than dyed unmordnated fabrics (Rather *et al.*, 2016a, 2016b).

4.8 Characteristics of Dyed Cellulosic Fabrics

The *B. vulgaris* peels and pomace dyed-mordanted cotton fabrics were of deeper shade than unmordanted dyed fabric as observed in Table 4.23 and Appendix 12. Deeper shades were observed in post-mordanted dyed fabrics while pre-mordanted dyed fabrics had the lightest shades among the dyed-mordanted cotton fabrics, confirming Yusuf *et al.* (2016) findings. This observation was similar to the findings of Bukhari *et al.* (2017), in dyeing of natural protein fibre with brown naphtoquinone dye extracted from *Juglans regia* L. alongside applying different metal salt mordants and evaluation of colour fastness properties. In general, the tannic acid-alum and $FeSO_4$ mordanted dyed fabrics

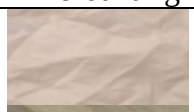
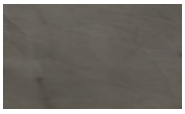
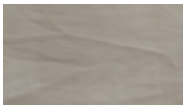
were outstanding in depth of shade than their corresponding $CuSO_4$ and $K_2Cr_2O_4$ dyed cellulosic fabrics (Table 4.23). Alum is easily available hence cheap, and safe to use mordant. Alum and chrome belongs to the group of brightening mordants. Therefore they usually produce pale versions of the dye colour in consideration. In Table 4.23, the tannic acid-alum mordanted fabric appears in the same hue but deeper shade as the *B. vulgaris* peels and pomace dye extract. Tannin that has a high affinity for cellulose is mixed with alum to help in the bonding process. Aluminum has a valency of three(+3) hence there is possibility that three molecules of dye is attached to each atom of the metallic mordant resulting in deeper and brighter staining characteristics (Asif, Ali, Saleem, & Hussain, 2010; Yusuf *et al.,* 2016). Consequentially, there is higher reactivity of Al^{3+} towards hydroxyl functional group (OH^-) of cellulosic substrate that accelerates diffusion of dye molecule into the fabric matrix (Arora *et al*., 2017). *B. vulgaris* is highly stable in acid media and this explains the persistent deeper shades in tannic acid-alum mordants.

$FeSO_4$ and $CuSO_4$ belongs to the dulling mordants (Konar & Samanta, 2011) group and produces dark or brown shades with distorted hue and depth from the dye in consideration as seen in Table 4.23. Due to their +2 valencies, two molecules of the natural dye is bonded to each atom of the mordant metal resulting in dull shades with distorted hues (Yusuf *et al.,* 2016).

However, the metallic mordants in the present study should be used in textiles for studies and comparative purposes only as most of them are toxic and carcinogenic in real life. (Fe), copper (Cu), chromium (Cr), vanadium (V) and cobalt (Co) owing to their rich coordination chemistry and redox properties undergo redox-cycling reactions, involving the Fenton-like production of superoxide anion, hydrogen peroxide and hydroxyl

radicals. These properties distort the body mechanisms such as transport, homeostasis, compartmentalization, tissue binding and cell constituents (Hegde *et al*., 2017). These metals bind to protein sites displacing those tailored for that purpose or competing with other metals in their natural binding sites. In particular, due to their neurotoxicity, hepatotoxicity and nephrotoxicity, they are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules causing cancers and other terminal ailments (Hegde *et al*., 2017). Unlike silk and wool, cotton lacks amino and carboxyl groups that could provide attachment sites to dye molecules (Arora *et al.*, 2017).

Table 4.23. Effect of mordants on shades of *B. vulgaris* peels and pomace dye extract.

Mordant	Pre mordanting	Simultaneous mordanting	Post mordanting
Without mordant			
Copper Sulphate			
Ferrous Sulphate			
P. dichromate			
Tannic acid-alum			
Reactive Orange HER			

4.8.1 FT-IR Characterization of Dyed and Mordanted Cotton Fabrics

The bleached and optimumly dyed–mordanted cotton fabrics were analysed by FTIR spectroscopy to ascertain structural changes due to the dye and mordants effects. The peaks at 2932cm^{-1} observed in Figure 4.11 Spectra (1) and (2) corresponds to asymmetric and symmetric stretching of long methylene (–CH_2–) chain for wax remnants on cotton fabric (Talari *et al.,* 2017). The stretching of betanin hexagonal rings and the C-C bonds within the rings gives rise to bands at 1045.33 cm^{-1}, 986.25 cm^{-1} and 918.67 cm^{-1} with sharp peaks (Galvan *et al.,* 2013). The strong band at 1045-986 cm^{-1} is possibly due to C–O–C symmetric stretching of dialkyl ether linkages and C–O stretching vibration for cellulose, hemicellulose and lignin. The band at 1588.42 cm^{-1} could be as a consequence of the extension stretching vibration of the C = N bond, that is aldimine bond that binds the betalamic acid with the dopa cycle in *B. vulgaris* peels and pomace dye extract on the cotton fabric. C–H wagging of lignin and carbohydrate in the fabric and dye sample are responsible for peak at 1342 cm^{-1} (Szymanska-Chargot *et al*., 2015). Peaks at 1045.33 cm^{-1} corresponds to asymmetric –C–O–C– ester linkage (Fahey, Nieuwoudt, & Harris, 2017); Huck, 2015; Szymanska-Chargot *et al*., 2015).

Spectra (1) and (2) in Figure 4.11 shows bleached fabric (1) and dyed cotton fabric with *B. vulgaris (*2). There are two significant changes resulting in higher bands at 3282.78 cm^{-1} and 1588.42 cm^{-1} for the spectra of dyed fabric (2) than bleached fabric (1). The higher absorbance units leading to higher bands at 3282.78 cm^{-1} are attributed to the stretching vibration of the –OH bond (OH wagging in phenolic compounds) according to Kumar *et al*. (2017). The 1588.42 cm^{-1} bands were most likely related to vibrational stretching of C = N groups as a result of aromatic ring deformations (stretching of

aromatic C=C). These are ascribed to alkaloids, flavonoids, tannins and lignin (de Assis *et al*., 2014) in the dye imparted on the fabric. There is a signal at 1624 cm^{-1} associated with the substantial extension of the aldimine bond that is part of the betanin molecule not identified.

The band at 3282.78 cm^{-1} is assigned to OH wagging (OH of phenolic compounds). The dyed cotton fabric with *B. vulgaris* peels and pomace (2) had two significant changes appearing at 3282.78 cm^{-1} respectively, which confirms the investigation of Basak *et al*. (2015) and de Assis *et al.* (2014) who found similar OH waggings in cellulosic fibres and banana fibres respectively after their surface modifications.

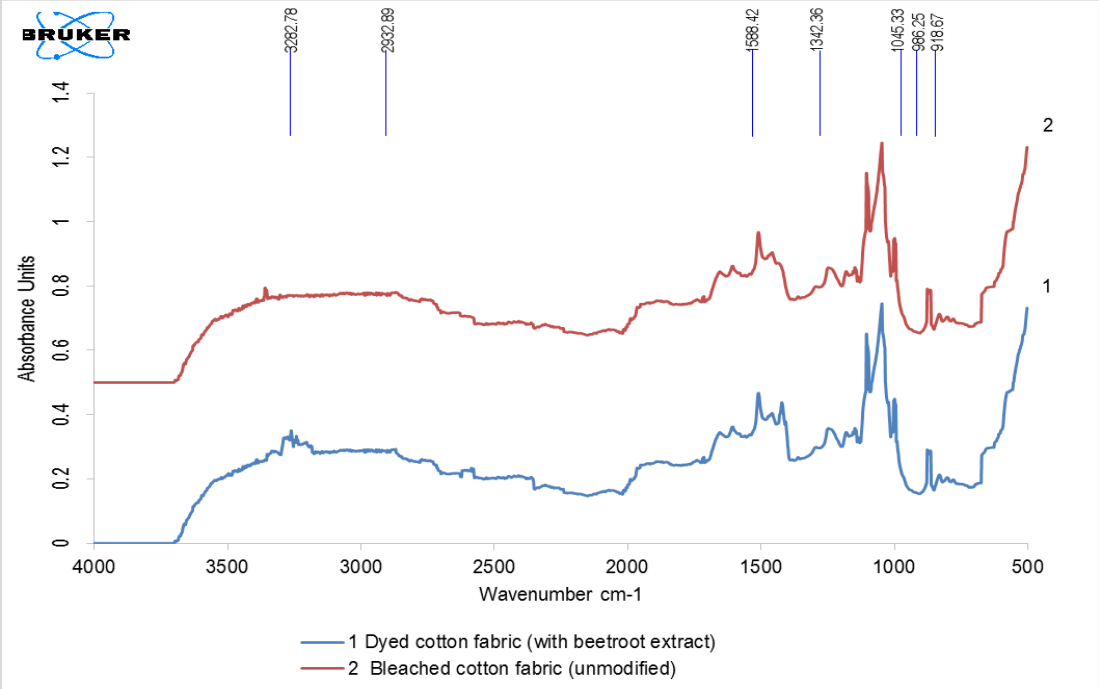


Figure 4.11. FTIR spectrum of bleached unmodified cotton fabric and cotton fabric dyed with *B. vulgaris* peels and pomace extract.

Characteristic bands were observed for different metal mordants as well as natural mordant, alum-tannic combination in wavenumbers ranging 4000–500 cm^{-1}. Figure 4.12 represents the IR spectra of dyed specimens after mordanting process. Peaks at 1371.89,

1025.23 and 985.28 cm^{-1} corresponds to –C–H bend (CH and aromatic vibrations), C-O-C deformation band (symmetric and asymmetric stretch due to primary and secondary alcohols) and stretching vibration of CH_2 respectively may be due to methanol solvent respectively (de Assis *et al.*, 2014), occurring as a result of linkages between the dye, mordant and cotton fabric.

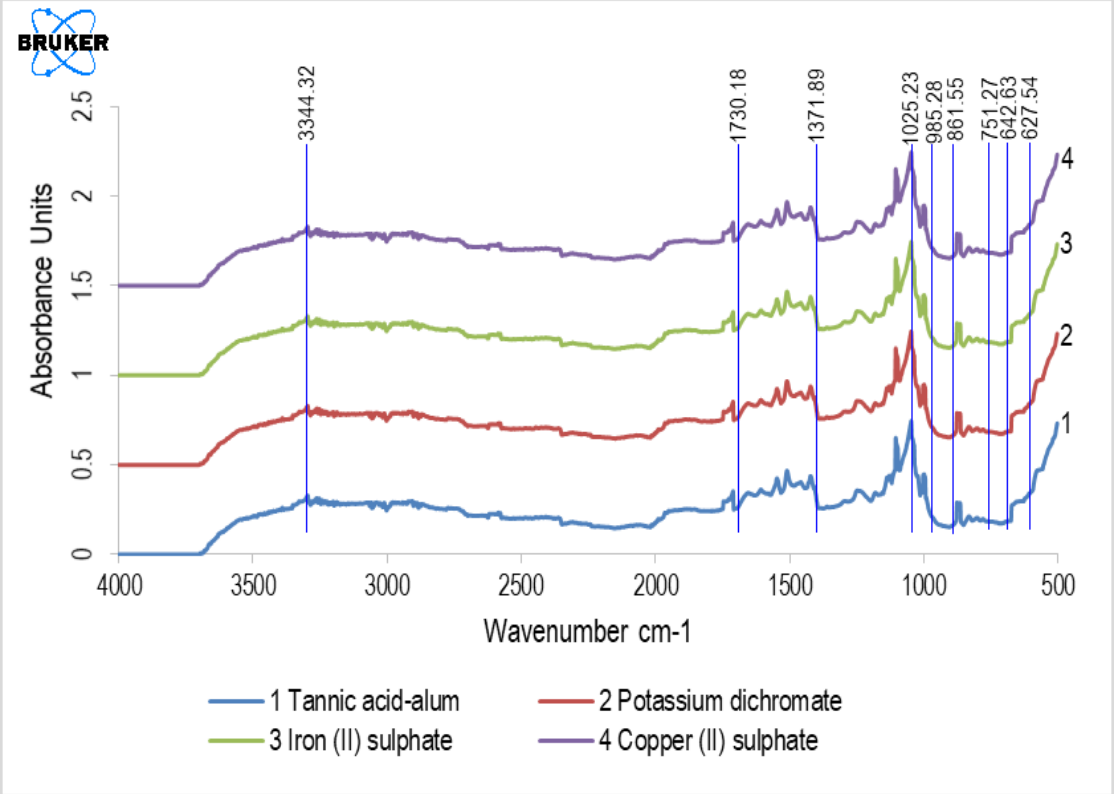


Figure 4.12. FTIR spectrum of the optimally dyed cotton fabric modified with natural mordant (1) and metallic mordants (2, 3 and 4).

Mordanting can cause spectral shift of diagnostic bands at the natural dye due to vibronic coupling between the cotton fabric, mordant and dye complex. Some mordants are dulling agents, thus they produce lighter colour hues, i.e. reflectance spectra with lower slopes. In Figure 4.12, there were some peak distortion to longer wavelengths

(bathochromic shift) in the spectra at 3344.32 cm^{-1} from 3282 cm^{-1} band owing to OH stretching as mordant combine with cellulose probably due to the presence of water and salts used in wet- processes (Abidi *et al.,* 2014). This bandwidth produced explains the darker purple-red hues of *B. vulgaris* peels and pomace extracts as their area and intensity increase explaining the presence of chromophores in the plant extracts. The influence of vibrational characteristics on the electronic properties of organic dye molecules includes changes in electron delocalization (Venkateshvaran *et al.,* 2014), that in turn affects the capacity of dye molecules to stabilize, that is scavenge for free radicals found in mordants such as metallic mordants and the degree of chemical interaction between fibre and dye (Venkateshvaran *et al.,* 2014).

The existence of chemistry in terms of covalent and coordinate bonds as well as complex dye formation make alum-tannic acid mordanted dyed cellulosic fabrics to have best fastness properties. Figure 4.13 depicts a plausible mechanism of interaction between the cotton fabric, mordants and betanin in *B. vulgaris* peels and pomace plant extract. The presence of substituent OH groups at the aromatic rings in the dye confers water solubility and enhances colour formation on *B. vulgaris* peels and pomace dye (Yusuf *et al.,* 2016).

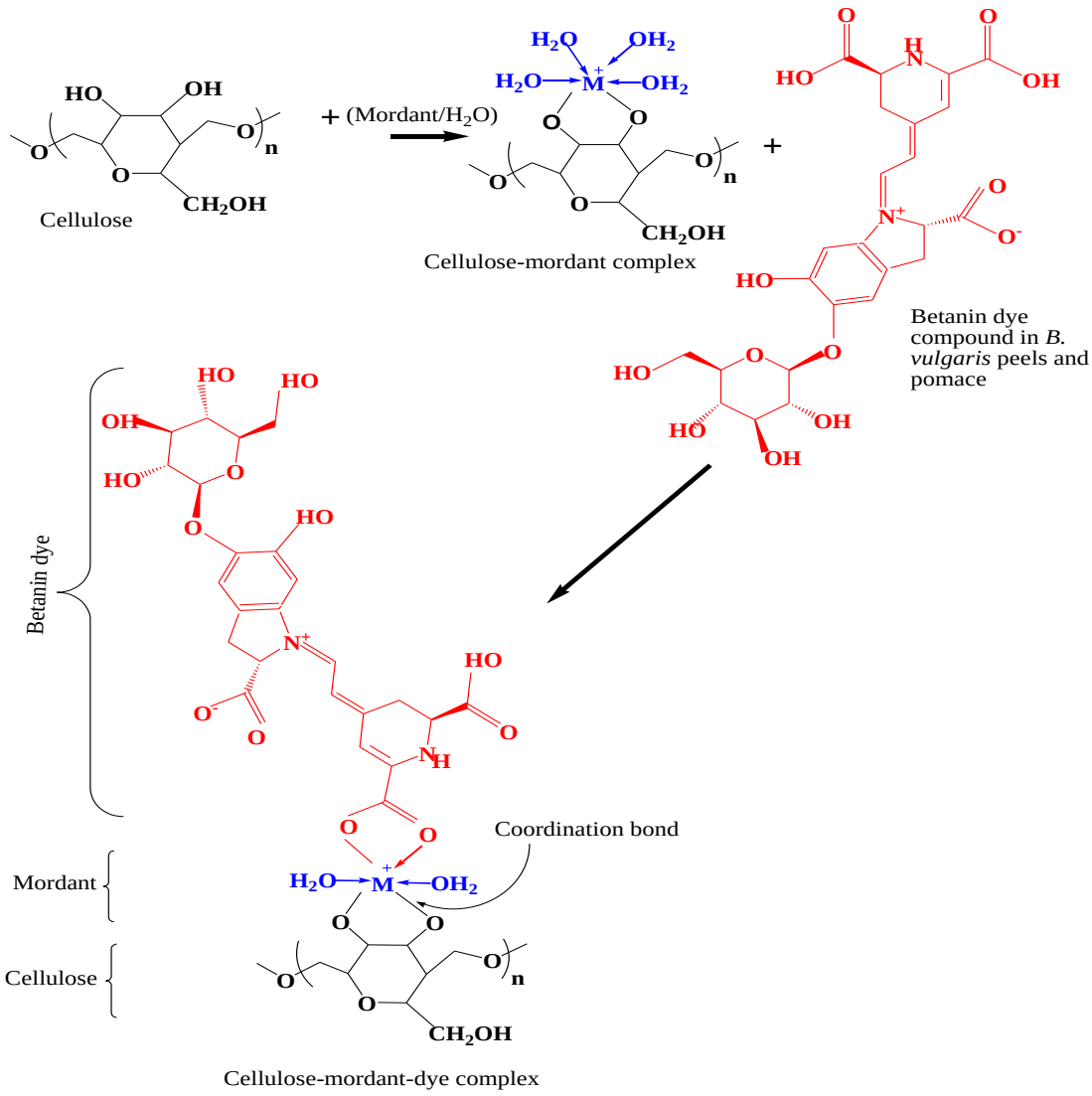


Figure 4.13. A plausible interaction of cotton fibre-mordant-betanin dye complex. M represents the metal ion in the mordant.

Dye molecules aggregation, together with covalent and coordinate bonds conferring good fastness properties on the dyed fabric. These observations strongly agreed with Geelani, Ara, Mir, Bhat, & Mishra (2016) findings in studies of dyeing and fastness properties of *Quercus robur dye* applied with natural mordants on natural fibres.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Extraction conditions of a natural dye from *B. vulgaris* were optimized in this study. The response surface methodology (RSM) and central composite design (CCD) used in this study showed that there exist a linear relationship among the extraction conditions (optimized extraction time of 11 hours and material to solvent ratio of 1:10 w/v) and that their response is directly proportional to percentage yield and the quantity of betalains in *B. vulgaris* peels and pomace extract.

Colourimetric properties of absorbance of the dye extract by UV–Vis spectroscopy provided two major absorption peaks at 538 and 492 nm demonstrating the presence of chromophores in the extract and synthetic dye respectively. The absorption peak observed at 538 nm as maximum absorbance wavelength (λ_{max}) was attributed to the presence of betanin in the extract, which is the major pigment in the *B. vulgaris* peels and pomace extract responsible for its red-purple colour. The FT-IR spectra further provided novel phytochemical markers from the different absorption bands characteristic of functional groups in *B. vulgaris* peels and pomace, in particular nitrogen containing compounds as seen by extension stretching vibration of the C = N bond at 1588.91 cm^{-1} band probably ascribed to molecules such as betanin.

The HPLC-UV and LC-MS with the aid of a betanin standard (betanidin 5-β-D-glucopyranoside) successfully qualified one outstanding peak at retention time of 7.699 min from the *B. vulgaris* peels and pomace sample, and through comparison of retention

times it closely corresponded to that of the standard (retention time of 7.6 min). Betanin was further established to be contributing approximately 38.16% of total betalain content in *B. vulgaris* peels and pomace.

Dyeing optimization by RSM and CCD provided suitable experimental design and regression model to achieve optimum dyeing conditions (temperature of 55 °C, time of 75 minutes and pH 6) that ensured better colouration results of cotton fabric and avoiding discrepancy in the dyed fabric quality. The study revealed that different shades of colour could be obtained in cotton dyeing while under treatment of metallic and natural mordants. Relative colour change (ΔE) and colour strength (K/S) were more enhanced by the application of post-mordanting methods across all mordants. This proved post – mordanting method as a better method of mordanting. The use of mordants generally improved the fastness properties of the cotton *B. vulgaris* peels and pomace dyed fabric. The color strength equivalence (Ceq) and color mass equivalence (Meq) of reddish *B. vulgaris* peels and pomace dye extract compared to synthetic Reactive Orange HER indicated that it has a considerable potential for application as a source of natural dye for cotton dyeing, hence solving the problem of waste disposal and providing a more sustainable option.

5.2 Recommendations

There is need for more research on ways to develop increased efficiency of the *B. vulgaris* peels and pomace natural dye for deeper absorption and maintain its reddish shade on cotton fabrics. Additionally, more advanced studies is needed for the natural dye. For instance ways of maintaining its original hue as physically observed in the dye source (red *B. vulgaris* peels and pomace) and development of spectral data for shade

reproduction. The dye in consideration has potential to be commercialized, so it must conform to the same stringent standards of performance that are applied to synthetic dyes.

REFERENCES

Abidi, N., Cabrales, L., & Haigler, C. H. (2014). Changes in the cell wall and cellulose content of developing cotton fibers investigated by FTIR spectroscopy. *Carbohydrate Polymers*, *100*, 9‑16.

Adeel, S., Bhatti, I. A., Kausar, A., & Osman, E. (2012). Influence of UV radiations on the extraction and dyeing of cotton fabric with Curcuma longa L. *Indian J. Fibre Text Res*., *37*, 87–90.

Ado, A., Yahaya, H., Kwalli, A., & Abdulkadir, R. S. (2014). Dyeing of Textiles with Eco-Friendly Natural Dyes: A Review. *International Journal of Environmental Monitoring and Protection*, *1*(5), 76-81.

Ai, Y. J., Tian, G., Liao, R. Z., Zhang, Q., Fang W. H., & Luo, Y. (2011). Intrinsic property of flavin mononucleotide controls its optical spectra in three redox states, *ChemPhysChem*, *12*, 2899-2902.

Almahy, H. A., & Ali, A. A. (2013). Extraction of carotenoids as natural dyes from the *Daucus carota* Linn (carrot) using ultrasound in Kingdom of Saudi Arabia. *Research Journal of Chemical Sciences*, *3*(1), 63–66.

Ali, N. F, & El-Mohamedy, R. S. R. (2011). Eco-freindly and protective natural dye from red prickly pear (opuntia Lasiacantha Pfeiffer) plant. *Journal of Saudi Chemical Society*, *15*, 257-261.

Ali, M. K., Islam, S., & Mohammad, F. (2016). Extraction of natural dye from walnut bark and its dyeing properties on wool yarn. *Journal of Natural Fibers*, *13*, 458–469.

Alsehri, S. M., Naushad, M., Ahamad, T., Alothman, Z.A, & Aldalbahi, A. (2014). Synthesis, characterization of curcumin based ecofriendly antimicrobial bio-adsorbent for the removal of phenol from aqueous medium. *Chem Eng J*, *254*,181–189.

Ambrogelly, A., & et al. (2018). Analytical comparability study of recombinant monoclonal antibody therapeutics, *10*, 513–538.

Antigo, J. L., Rita de Cássia Bergamasco, R., & Grasiele, S. M. G. S. (2017). Effect of pH on the stability of red beet extract (*Beta vulgaris* L.) microcapsules produced by spray drying or freeze drying. *Food Sci. Technol*, *.38*, 1678-457.

Arora, J., Agarwal, P., & Gupta, G. (2017). Rainbow of natural dyes on textiles using plants extracts: Sustainable and eco-friendly processes. *Green and Sustainable Chemistry*, *7*(01), 35-47.

Asif, A., Ali, S., Saleem, H., & Hussain, T. (2010). Effect of Tannic Acid and Metallic Mordants on the Dyeing Properties of Natural Dye Extracted from Acacia nilotica bark. *Asian Journal of Chemistry, 22*(9), 7065- 7069.

Azeredo, H. M. (2009). Betalains Properties, sources, applications, and stability – a review. *International Journal of Food Science & Technology*, *44*, 2365-2376.

Baião, D., & Silva, D., Mere Del Aguila, E. (2017). Paschoalin V. Nutritional, Bioactive and Physicochemical Characteristics of Different Beetroot Formulations.

Banerjee, S., & Sharma, Y. C. (2013). Equilibrium and kinetic studies for removal of malachite green from aqueous solution by a low cost activated carbon. *J Ind Eng Chem*, *19*(4), 1099–1105.

Banu, K. S., & Cathrine, L. (2015). General techniques involved in phytochemical analysis. *International journal of advanced research in chemical science*, *2*(4), 25-32.

Basak, S., Samanta, K. K., Arputhraj, A., Saxena S, Mahangade, R., & Narkar, R. (2012). Method of dyeing and protective finishing of cotton textiles using vegetable extract.

Basak, S., Samanta, K., Saxena, S., Chattopadhyay, S. K., Narkar, R., Mahangade, R., & et al. (2015). Flame resistant cellulosicsubstrate using banana pseudostem sap. *Polish Journal of Chemical Technology, 17*(1), 123–133.

Bhuiyan, M. A. R., Islam, A., Islam, S., Hossain, A., & Nahar, K. (2017). Improving dyeability and antibacterial activity of *Lawsonia inermis* L. on jute fabrics by chitosan pretreatment. *Textile and Clothing Sustainability*, *3*(1), 1–10.

Bru¨schweiler, B. J., & Merlot, C. (2017). Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. *Regul Toxicol Pharmacol*, *88*,214–226.

Bukhari, M. N., Islam, S., Shabbir, M., Rather, L. J., Shahid, M., & Singh, U. (2017). Dyeing studies and fastness properties of brown naphtoquinone colorant extract from *Juglans regia* L. on natural protein fibre using different metal salt mordants. *Textiles and Clothing Sustainability*, *3*(3), 1–9.

Burkinshaw, S. M. D. S., Jeong, D. S., & Chun, T. I. (2013). *Dyes and Pigments, 97*, 374–387.

Cerezo, J., Zúñiga, J., Requena, A., Ávila Ferrer, F. J., & Santoro, F. (2013). Harmonic models in cartesian and internal coordinates to simulate the absorption spectra of carotenoids at finite temperatures, *J. Chem. Theory Comput.*, *9*, 4947-4958.

Chaudhry, Siddiqui, S. I., Fatima, B., Tara, N., Rathi, G., & Chaudhry, S. A. (2019). Recent advances in remediation of synthetic dyes from wastewaters using

sustainable and low-cost adsorbents. *The impact and prospects of green chemistry for textile technology*, pp. 471-507.

Chawla, H., Parle, M., Sharma, K., & Yadav, M. (2016). Beetroot: A Health Promoting Functional Food. *Nutraceuticals, 1,* 0976-3872.

Chernetsova, E. S., & Morlock, G. E. (2012). Assessing the capabilities of direct analysis in real time mass spectrometry for 5-hydroxymethylfurfural quantitation in honey. *International Journal of Mass Spectrometry*, 314, 22–32.

Chhikara, N., Devi, H. R., Jaglan, S., Sharma, P., Gupta, P., & Panghal, A. (2018). Bioactive compounds, food applications and health benefits of Parkia speciosa (stinky beans): a review. *Agriculture & Food Security, 7(1),* 46.

Clark, M. (2011). Handbook of Textile and Industrial Dyeing: Principles, *Processes and Types of Dyes*.

Clifford, T., Howatson, G., West, D. J, & Stevenson, E. J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients, 7*(4), 2801-2822.

Costa, A. F. S, Rocha, M. A. V, & Sarubbo, L. A. (2017). Bacterial cellulose: an ecofriendly biotextile. *International Journal of Textile and Fashion Technology*, *7*, 11-26.

de Assis, F., Margem, F., Loiola, R., & Monteiro, S. (2014). Characterization of banana fibres functional groups by infrared spectroscopy. *Materials Science Forum, 250,*775–776.

Degano, I., Biesaga, M., & Colombini, M. P. (2011). Trojanowicz, M.Historical and Archaeological Textiles: An Insight on Degradation Products of Wool and Silk Yarns. *J. Chromatogr., 1218*, 5837–5847.

Djelal, H., Corn´ee C., Tardivel, R., Lavastre O., & Amrane, A. (2013). The use HPTLC and Direct Analysis in Real Time-Of-Flight Mass Spectrometry DART-TOF-MS for rapid analysis of degradation by oxidation and sonication of an azo dye. *Arabian Journal of Chemistry*.

Devi Rajeswari, V. *et a*l. (2015). Extraction of natural dye from marigold flower (Tageteserectal.) and dyeing of fabric and yarns: A focus on colorimetric analysis and fastness properties. *Der Pharmacia Lettre, 7* (1),185-195 .

Ding, Y. I., & Freeman, H. S. (2017). Mordant dye application on cotton; optimization and combination with natural dyes. *Coloration Technology*, *133*(5), 369–375.

El-Beltagi, H. E. S. (2011). Effect of roasting treatments on protein fraction profiles, some enzyme activities of Egyptian peanuts. *Int. J. Food. Sci. Nutr*. 62, 453-456.

Esatbeyoglu, T., Wagner, A. E., Schini-Kerth, V., & Rimbach, G. (2015). Betanin – A food colorant with biological activity.*Molecular Nutrition & Food Research*, *59*, 36–47.

Espinosa, M. Y., Reyes J., Hermosín, B., & Azamar-Barrios, J. A. (2012). Characterisation of a Natural Dye by Spectroscopic and Chromatographic Techniques. *Masterial Research Society. Symp. Proc., 1374*, 1-11.

Fahey, L. M., Nieuwoudt, M. K., & Harris, P. J. (2017). Predicting the cell-wall compositions of Pinus radiata (radiata pine) wood using ATR and transmission FTIR spectroscopies. *Cellulose, 24*(12), 5275‑5293.

Francine, U., Jeannette, U., & Pierre, R. J. (2015). Assessment of antibacterial activity of neem plant (Azadirachta indica) on Staphylococcus aureus and Escherichia coli. *Journal of Med Plants, 3*(4), 85-91.

Frose, A., Schmidtke, K., Sukmann, T., Junger, I. J., & Ehrmann, A. (2019). Application of natural dyes on diverse textile materials. *International Journal for Light and Electron Optics, 181*(2018), 215–219.

Galván, J. A., Solano, F., & Wakamatsu, K. (2013). Vibrational characterization of pheomelanin and trichochrome F by Raman spectroscopy, *Spectrochim. Acta A, 110*, 55-59.

Garg, N., Kapono, C. A., Lim, Y. W., Koyama, N., Vermeij, M. J., Conrad, D., Rohwer, F., & Dorrestein, P. C. (2015). Mass spectral similarity for untargeted metabolomics data analysis of complex mixtures. *Int. J. Mass Spectrom*, *377*, 719–727.

Geelani, S. M., Ara, S., Mir, N. A., Bhat, S. J. A., & Mishra, P. K. (2016). Dyeing and fastness properties of *Quercus robur* with natural mordants on natural fibre. *Textile and Clothing Sustainability, 2*(8), 1–10.

Georgouli, K., Del Rincon, J. M., & Koidis, A. 2017. Continuous statistical modelling for rapid detection of adulteration of extra virgin olive oil using mid infrared and Raman spectroscopic data. *Food Chem.*, *217*:735–742.

Ghaheh, F. S., Nateri, A. S., Mortazavi, S. M., Abedi D. & Mokhtari, J., (2012).*Color. Technol.*, 128-473.

Ghouila, H., Meksi, N., Haddar, W., Mhenni, M., & Jannet, H. (2012). Extraction, identification and dyeing studies of Isosalipurposide, a natural chalcone dye from Acacia cyanophylla flowers on wool. *Industrial Crops and Products, 35*,31-36. pp 70.

Gokhale, S. V., & Lele, S. S. (2014). Betalain content and antioxidant activity of *Beta vulgaris*: Effect of hot air convective drying and storage. *Journal of Food Processing and Preservation*, *38*, 585–590.

Gordon, B. (2015). *Dye bath Monitoring*. North Carolina: College of Textiles, North Carolina State University. Retrieved December, *15*, 2015.

Guldiken, B., Toydemir, G., Nur Memis, K., Okur S., Boyacioglu, D., & Capanoglu, E. (2016). Home-processed red beetroot (Beta vulgaris L.) products: changes in antioxidant properties and bioaccessibility. *Int J Mol Sci*., *17*(6)-858.

Hamanthraj, K. P. M., Desai, S. M., & Bisht, S. S. (2014). Optimization of Extraction Parameters for Natural Dye form Pterocarpus Santalinus by Using Response Surface methodology. *International Journal of Engineering Research and Applications, 4*(9), 100- 108.

Hatlestad, G. J., Sunnadeniya, R. M., Akhavan, N. A., Gonzalez, A., Goldman, I. L., McGrath, J. M., & Lloyd, A. M. (2012). The beet R locus encodes a new cytochrome P450 required for red betalain production. *Nature Genetics, 44*, 816–820.

Hegde, R. P., Fedorov, A. A., Sauder, J.M., Burley, S.K., Almo, S.C., Ramagopal, U.A. (2017). The hidden treasure in your data: Phasing with unexpected weak anomalous scatterers from routine data sets. *Acta Cryst. F Struct. Biol. Commun*., *73*, 18.

Hong, K. H. (2018). Effects of tannin mordanting on coloring and functionalities of wool fabrics dyed with spent coffee grounds. *Fashion and Textiles, 5,* 33.

Huck, C. W. (2015). Advances of infrared spectroscopy in natural product research. *Phytochemistry Letters*, *11*, 384‑393.

Jacquemin, D., Brémond E., Ciofini I., & Adamo, C. (2012). Impact of vibronic couplings on perceived colors: two anthraquinones as a working example, *J. Phys. Chem. Lett.*, 3, 468-471.

Jain, H. (2013). Dyeing of cotton fabrics with extract of jamun tree and its by-products using natural mordants. *Coulourage*, 40-43.

Kathiravan, T., Nadanasabapathi, S., & Kumar, R. (2014). Standardization of process condition in batch thermal pasteurization and its effect on antioxidant, pigment and microbial inactivation of Ready to Drink (RTD) beetroot (Beta vulgaris L.) juice. *International Food Research Journal, 21*(4), 1305.

Kaur, K., Kumar, B., Garg, N., & Navneet, K. (2014). Statistical Optimisation of Conditions for Decolourisation of Synthetic Dyes by Cordyceps militaris MTCC 3936 Using RSM. *BioMed Research International, 2015*, 17.

Kechi, A., Chavan, R., & Moeckel, R. (2013). Dye Yield, Color Strength and Dyeing Properties of Natural Dyes Extracted from Ethiopian Dye Plants. *Textiles and Light Industrial Science and Technology, 2*(3), 137-145.

Khan, A., Hussain, M. T., & Jiang, H., *et al*. (2018a). Development of functional wool fabric by treatment with aqueous and alkaline extracts of Cinnamomum camphora plant leaves. *J Nat. Fiber*, 1–10.

Khan, A., Hussain, M. T. & Jiang, H. (2018b). Dyeing of silk fabric with natural dye from camphor (Cinnamomum camphora) plant leaf extract. *Coloration Technology*. doi:10.1111/cote.12338.

Khan, A., Iqbal, N., Adeel, S., Azeem, M., Batool, F., Bhatti, A. (2014). Extraction of natural dye from red calico leaves: gamma ray assisted improvements in colour strength and fastness properties. *Dyes Pigments*, *103*, 50–54.

Khan, M. I., Harsha, P. S. C. S., Giridhar, P., & Ravishankar, G. A. (2012). Pigment identification, nutritional composition, bioactivity, and *in vitro* cancer cell cytotoxicity of *Rivina humilis* L. berries, potential source of betalains. *LWT - Food Sci Technol*., *47*(2), 315–23.

Khandegar, V., & Saroha, A. K. (2013). Electrocoagulation for the treatment of textile industry effluent–a review. *J Environ Manag*, *128*,949–963.

Kljun, Alenka & El-Dessouky, Hassan & Benians, Thomas & Goubet, Florence & Meulewaeter, Frank & Knox, Paul & Blackburn, Richard. (2014). Analysis of the physical properties of developing cotton fibres. *European Polymer Journal. 51*. 57–68. 10.1016.

Konar, A., Samanta, A. K. (2011). *Dyeing of Textiles with Natural Dyes.* India.

Kopsell, D. A., Sams, C. E., Deyton, D. E., Abney, K. R., David, K. E., Robertson, L. (2010). Characterization of nutrionally important carotenoids in Buching Onion. *Hort Science, 45*(3), 463- 465.

Kulkarni, S. S., Gokhale, A. V., Bodake, U. M., & Pathade, G. R. (2011). Cotton dyeing with natural dye extracted from pomegranate (*Punica granatum*) Peel. *Univers J Environ Res Technol*, *1*(2), 135–139.

Kumar, S. N. A., Ritesh, S. K., Sharmila, G., & Muthukumaran, C. (2017). Extraction optimization and characterization of water soluble red purple pigment from floral bracts of *Bougainvillea glabra*. Arab J Chem, *10*, 2145–2150.

Kumar, S., & Su-Ling Brooks, M., (2018). Use of red beet (Beta vulgaris L.) for antimicrobial applications—; a critical review. *Food and Bioprocess Technology*, *18*, 17–42.

Kumaresan, M., Palanisamy, P. N., & Kumar, P. E., (2011). Application of Eco-friendly Natural dye obtained from flower of Spathodea campanulata on silk using the combination of mordants. *European Journal of Scientific Research*, *52*(3), 306-312.

Kumorkiewicz, A. (2017). Thermal degradation of major gomphrenin pigments in the fruit juice of Basella alba L. (Malabar Spinach). *J Agric Food Chem*, *65*, 7500–7508. jafc.7b02357.

Kusumawati, N., Santoso, A. B., Sianita, M. M., & Muslim, S. (2017). Extraction, Characterization and Application of Natural Dyes from the Fresh Mangosteen (Garcinia mangostana L.) Peel. International Journal on Advanced Science, *Engineering and Information Technology, 7*(3), 878-884.

Lakshmi, C. (2015). Impact of Textiles and Clothing Industry on Environment: Approah towards eco-friendly textiles. *Bangalore: Fibre2Fashion Pvt.*

Lech, K., & Jarosz, M. (2016). Identification of Polish Cochineal (Porphyrophora Polonica L.) in Historical Textiles by High-Performance Liquid Chromatography Coupled with Spectrophotometric and Tandem Mass Spectrometric Detection. *Anal. Bioanal. Chem*., *408*, 3349–3358.

Lee, E. J., An, D., Nguyen, C. T. T., Patil, B. S., Kim, J., & Yoo, K. S. (2014). Betalain and betaine composition of greenhouse- or field-produced beetroot (Beta vulgaris L.) and inhibition of HepG2 cell proliferation. *Journal of Agricultural and Food Chemistry, 62*, 1324–1331.

Li, H. X., Xu, B., Tang, L., Zhang, J. H., & Mao, Z. G. (2015). Reductive decolorization of indigo carmine dye with Bacillus sp. MZS10. *International Biodeterioration & Biodegradation*, *103*, 30-37.

Li, P., Yin ZQ, Li SL, Huang XJ, Ye WC, Zhang QW. (2014). Simultaneous determination of eight flavonoids and pogostone in Pogostemon cablin by high performance liquid chromatography. *J Liq Chromatogr Relat Technol*.; *37*(12), 1771–84.

Lidder, S., & Webb, A. J. (2013). Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. *British Journal of Clinical Pharmacology, 75*, 677–696.

Lim, A., Damit, D. N. F. B., & Ekanayak, P. (2015). Tailoring of extraction solvent of *Ixora coccinea* flower to enhance charge transport properties in dye-sensitized solar cells. *Ionics, 21,* 2897–2904.

Lioe, H. N., Adawiyah, D. R., & Anggraeni R. (2012). Isolation and Characterisation of the major natural dyestuff component of Brazilwood (Caesalpinian sappon L). *International Food Research Journal*, *19*(2), 537- 542.

Lokesh, P., & Kumara, S. M. (2013). Extraction of natural dyes from Spathodea campanulata and its application on Silk and Cotton Fabrics. *Journal of Der Chemica Sinica, 4*(1), 111-115.

Makkar, P., Singh, S. S., & Rose, N., (2013). Color fastness of tesu dyed silk. *Research Journal of Family, Community and Consumer Sciences*, *1*, 1-5.

Manasa, M., Sindiri, M. K., & Vangalapati, M. (2013). Optimisation of physico-chemical parameters for the extraction of flavonoids and phenolic compounds from the skin of Allium capa. *International Journal of Innovative Research in Science, Engineering and Technology, 2*(7), 1-5.

Maraie, N. K., Abdul-Jalil, T. Z., Alhamdany, A. T., & Janabi, H. A. (2014). Phytochemical study of the Iraqi Beta vulgaris leaves and its clinical application for the treatment of different dermotological diseases. *World Journal of Pharmacy and Pharmaceutical Sciences, 3,* 05-19.

Măruţoiu, O., Gogoaşă, I., Măruţoiu, C., Tofană, M., Moigrădean, D., & Gergen, I. (2011). Separation and identification of some synthetic food colorants from foods through thinlayer chromatography – UV/VIS spectrometry. *J. Agroaliment. Proc. Technol*., *17*(1), 46-53.

Mikolajczyk-Bator, K., Blaszczyk, A., Czyżniejewski, M., & Kachlicki, P. (2016). Identification of saponins from sugar beet (Beta vulgaris) by low and high-resolution HPLC– MS/MS. *Journal of Chromatography B, 1029,* 36-47.

Mikołajczyk-Bator, K., & Pawlak, S. (2016). The effect of thermal treatment on antioxidant capacity and pigment contents in separated betalain fractions. *Acta Sci Pol Technol Aliment, 15*,257–265.

Mishuk, A. I., &Rahman, H. (2014). Impact of Pespiration on Finished Woven Fabrics used by Various Professional Personels. *BUBT Journal*, 97-101.

Mokhtari-Dehkordi, S., Hojjati, M., Rouhi, L., Rabiei, Z., & Alibabaei, Z. (2014). Effect of etha-nolic extract of beet roots and leaves on motor coordination in male Wistar rats. *J. Shahrekord Univ. Med. Sci, 16*, 14-23.

Molina, G. A., Herna´ndez-Martı´nez, A. R., Cortez-Valadez M., & et al. (2014). Effects of tetraethyl orthosilicate (teos) on the light and temperature stability of a pigment from beta vulgaris and its potential food industry applications. *Molecules*, *19*, 17985–18002.

Morgado, M., de Oliveira, G. V., Vasconcellos, J., Monteiro, M. L., Conte-Junior, C., Pierucci, A. P. T. R., et al. (2016). Development of a beetroot-based nutritional gel containing high content of bioaccessible dietary nitrate and antioxidants. *Int J Food Sci Nutr*, *67*(2), 153–60.

Moore, J. C., Spink, J., & Lipp, M. (2012). Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *J Food Sci*. *77*, 4.

Mroczek, A., Kapusta, I., Janda, B., & Janiszowska, W. (2012). Triterpene saponin content in the roots of red beet (Beta vulgaris L.) cultivars. *Journal of Agricultural and Food Chemistry*, *60*, 12397-12402.

Nasirizadeh, N., Dehghanizadeh, V., Yazdanshenas, M. E., Moghadam, M. R., & Karimi A. (2012). Optimization of wool dyeing with rutin as natural dye by central composite design method. *Industrial Crops and Products, 40*, 361-366.

Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T., & Wybraniec, S. (2011). Betalainic and nutritional profiles of pigment-enriched red beet root (Beta vulgaris L.) dried extracts. *Food Chemistry, 127*, 42-53.

Ninfali, P., Angelino, D. (2013). Nutritional and functional potential of *Beta vulgaris* cicla and rubra. F*itoterapia*, 2013 Sep; 89:188-99.

Nowak, C. & *et al*. (2017). Forced degradation of recombinant monoclonal antibodies: *A practical guide*, *9*, 1217–1230.

Nyquist, R. A., & Kagel, R. O. (2012). Handbook of infrared and Raman spectra of inorganic compounds and organic salts: infrared spectra of inorganic compounds. *London, UK: Academic Press*.

Odoh, U.E., & Okoro, E. C. (2013). Quantitative phytochemical, proximate/nutritive composi-tion analysis of *Beta vulgaris* linnaeus (cheno-podiaceae). *Int. J. Curr. Res, 5*(12), 3723-3728.

Panghal, A., Virkar, K., Kumar, V., Dhull, S. B., Gat, Y., & Chhikara, N. (2017). Development of Probiotic Beetroot Drink. *Current Research in Nutrition and Food Science Journal*, *5*.

Prabhavathi, P., Devi, A. S., & Anitha, A. (2014). Improving the sunlight fastness & wash fastness of the eucalptus bark dye on cotton. *Journal of Polymer and Textile Engineering, 1*(4), 27- 30.

Prabhu, & Bhute, A. S. (2012). Plant based natural dyes and mordants. *J. Nat. Prod. Plant Resource, 2* (6), 649-664.

Prieto-Santiago, V., Cavia, M. M., Alonso-Torre, S. R., Carrillo, C. (2020**).** Relationship between color and betalain content in different thermally treated beetroot products. *J. Food Sci. Technol.*, *57*, 3305–3313.

Pyrzynska, K., & Pekal A., (2011). Crit. Rev. Anal. Chem., *41*, 335. [19–21].

Rather, L. J., Islam, S., & Mohammad, F. (2015). Study on the application of Acacia nilotica natural dye to wool using fluorescence and FT-IR spectroscopy. *Fibers and Polymers*, *16*, 1497–1505.

Rather, L. J., Islam, S., Azam, M., Shabbir, M., Bukhari, M. N., Shahid, M., Khan, M. A., Haque, Q. M. R., & Mohammad, F. (2016). Antimicrobial and fluorescence finishing of woolen yarn with Terminalia arjuna natural dye as an ecofriendly substitute to synthetic Antibacterial agents. *RSC Advances*, *6*, 39080–39094.

Rather, L. J., Shabbir, M. N. Bukhari, M. Shahid, M. A. Khan, & F. Mohammad. (2016). Ecological dyeing of woollen yarn with Adhatoda vasica natural dye in the presence of biomordants as an alternative copartner to metal mordants. *Journal of Environmental Chemical Engineering*, *4*, 3041–49.

Ravichandran, K., Saw, N. M. M. T., Mohdaly, A. A., Gabr, A. M., Kastell, A., Riedel, H., & Smetanska, I. (2013). Impact of processing of red beet on betalain content and antioxidant activity. *Food Research International, 50,* 670-675.

Rawat, D., Mishra, V., & Sharma R. S. (2016). Detoxification of azo dyes in the context of environmental processes. *Chemosphere, 155,591*–605.

Razak, N. A, Tumin, S. M, & Tajuddin, R. (2011). Effect of Temeprature on the Colour of Natural Dyes extracted Using Pressurised Hot Water Extraction Method. *American Journal of Applied Sciences, 8*(1), 45 - 49.

Rebecca, J., Sharmila, S., Das, M. P., & Seshiah, C. (2014). Extraction and purification of carotenoids from vegetables. *Journal of Chemical and Pharmaceutical Research, 6,* 594-598.

Rosa, M. R, Garcı´a-Falco´n, M. S, Simal-Ga´ndara, J, Rodrigues, A. S, & Domingos, P. F. A. (2010). Identification and quantification of Flavonoids in traditional cultivators of red 121 and white onions at harvest. *Journal of Food Composition and Analysis, 23,* 592- 598.

Saha, P. D., & Sinha, K. (2012). Natural dye from Bixa Seeds as a Potential Alternative to Synthetic dyes for use in Textile Industry, Desalination and Water Treatment. *40,* 298-301.

Sampath, M., & Vasanthi, M. (2013). Isolation, structural elucidation of flavonoids from Polyathia longifolia and evaluation of antibacterial, antioxidant and anticancer potential. *International Journal of Pharmacy and Pharmaceutical Science,* 5(1), 336-341.

Sanjeeda, I., & Taiyaba, A. N. (2014). Natural Dyes: Their Sources and Eco-friendly Use as Textile Materials. *Journal of Environmental Research and Development, 8*(3), 683-687.

Sardana, R. K., Chhikara, N., Tanwar, B., & Panghal, A. (2018). Dietary impact on esophageal cancer in humans: a review. *Food & Function, 9,* 1967-1977.

Sarhan, T. M., & Salem, A. A. (2018). Turmeric dyeing and chitosan/titanium dioxide nanoparticle colloid finishing of cotton fabric. *Indian Journal of Fibre & Textile Research, 43*(4), 464-473.

Saxena, H. O, Tiwari, R., & Pandey, A. K. (2012). Optimization of Extraction & Dyeing Conditions of Natural Dyes from Butea monosperma (Lam) Kuntze Flowers and Development of Various Shades. *Environmental & We an International Journal of Science and Technology, 7,* 29-35.

Saxena, M., Saxena, J, Nema, R., Singh, D, & Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of pharmacognosy and phytochemistry*, *1*(6), 168-182.

Saxena, S., & Raja, A. S. M. (2014). Natural Dyes: Sources, Chemistry, Application and Sustainability Issues. In S. Muthu (Ed.), *Textiles Science and Clothing Technology* (pp. 37-80). India: Central Institute for Research on Cotton Technology.

Shahid, M., Ahmed, A., Yusuf, M., Khan, M. I., Khan, S. A., Manzoor, N., & et al. (2012). Dyeing, fastness and antimicrobial properties of woolen yarns dyed with gallnut (*Quercus infectoria* Oliv.) extract. *Dyes and Pigments, 95,* 53–61.

Shahid, M., Islam, U.I., & Mohammad, F., (2013). Recent advancements in natural dye applications. *J. Cleaner Prod*., *1*–22.

Shena, Y., van Beeka T. A., Claassena, F. W., Zuilhofa, H., Chenb, B., & Nielen M. W. F. (2012). Rapid control of Chinese star anise fruits and teas for neurotoxic anisatin by Direct Analysis in Real Time (DART) high resolution mass spectrometry. *Journal of Chromatography* A, *03*, 058.

Sengupta, D, Mondal B., & Mukherjee, K. (2015). Visible light absorption and photo-sensitizing properties of spinach leaves and beetroot extracted natural dyes. *Spectrochim Acta A Mol Biomol Spectrosc*, *148*, 85–92.

Serrano, A., Sousa, M. M., Hallett, J., Lopes, J. A., & Oliveira, M. C. (2011). Analysis of Natural Red Dyes (Cochineal) in Textiles of Historical Importance Using HPLC and Multivariate Data Analysis. *Anal. Bioanal. Chem*., *401*, 735–743.

Simpson, J. T., Hunter, S. R., & Aytug, T. (2015). Superhydrophobic materials and coatings: a review. *Reports on Progress in Physics*, *78*(8), 086501.

Singh, A., Ganesapillai M., & Gnanasundaram N. (2017). Optimization of extraction of betalain pigments from *Beta vulgaris* peels by microwave pretreatment. *IOP Conf Ser Mater Sci Eng, 26*3, 032004.

Sinha, K., Saha, P. D., & Datta, S. (2012). Response surface optimization and artificial neural networmodeling of microwave assisted natural dye extraction from pomegranate rind. *Ind. Crops Prod., 37*(1), 408–414.

Soheil, A. N., Abu Bakar M., & Norasikin A. L. (2017). The extraction and absorption study of natural dye from Areca catechu for dye sensitized solar cell application. *AIP Conference Proceedings, 1838*, 020019.

Slatnar, A., Stampar, F., Vebaric, R., & Jakopic, J. (2015). HPLC-MSn identification of betalains profile of different beetroot (Beta vulgaris L. ssp. vulgaris) parts and cultivars. *Journal of Food Science, 80,* 1952–1958.

Song, J. Y., Jing L., & Rui, D. (2012). Preparative isolation and purification of indigo and indirubin from *folium isatidis* by high-speed counter-current liquid chromatography. *Photochemical Analysis,* 23 (6), 637-641.

Stewart, C. W. (2017) Comparative Study of the Environmental Impacts of Standardized Dyeing Systems Using Natural and Synthetic Dyes on Knitted Cotton Fabric. *Fiber and Polymer Science, 3*(1), 76-84.

Sturzoiu, Aura & M., Stroescu & Stoica, Anicuta & Dobre, & Tănase. (2011). Betanine extraction from Beta vulgaris - Experimental research and statistical modeling. *UPB Scientific Bulletin, Series B: Chemistry and Materials Science, 73*.

Sufian, A., Hannan, A., Rana, M., & Huq, M. Z. (2016). Comparative study of fastness properties and color absorbance criteria of conventional and avitera reactive dyeing on cotton knit fabric. *European Scientific Journal, 12*(15), 352–364.

Sun, S. S., & Tang, R. C. (2011). Adsorption and UV protection properties of the extract from honeysuckle onto wool. Industrial & Engineering Chemistry Research, *50,* 4217–4224.

Szopinska, A. A., & Gawęda, M. (2013). Comparison of yield and quality of red beet roots cultivated using conventional, integrated and organic method. *Journal of Horticultural Research, 21,* 107-114.

Szymanska-Chargot, M., Chylinska, M., Kruk, B., & Zdunek, A. (2015). Combining FT-IR spectroscopy and multivariate analysis for qualitative and quantitative analysis of the cell wall composition changes during apples development. *Carbohydrate Polymers, 115,* 93‑103.

Talari, A. C. S., Martinez, M. A. G., Movasaghi, Z., Rehman, S., & Rehman, I. U. (2017). Advances in Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Applied Spectroscopy Reviews, 52*(5), 456‑506.

Tayade, P. B., & Adivarekar, R.V. (2013). Adsorption kinetics and thermodynamic study of Cuminum cyminum L. dyeing on silk. *J. Environ. Chem. Eng*., *1*(4), 1336–1340.

Tera, F. M, Elnagar, K. E, & Mohamed, S. M. (2012). Dyeability and Light Fastness Properties of Onion Scale Dye on Different FAbric Types for Conservation Applications. *Journal of Textile and Apparel, Technology and MAnagement, 7*(3), 1-6.

Ticha, M. B., Haddar, W., Meksi, N., *et al.* (2016). Improving dyeability of modified cotton fabrics by the natural aqueous extract from red cabbage using ultrasonic energy. *Carbohydr Polym, 154,* 287–295.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: A review. *Internationale Pharmaceutica Sciencia, 1*(1), 98-106.

Tutak, M., & Benli, H. (2012). Dyeing properties of textiles by Turkish hazelnut (Corylus colurna): leaves, coat, shell and dice. *Color. Technol.*, *6*, 454–458.

Uddin, M. G. (2015). Extraction of eco-friendly natural dyes from mango leaves and their application on silk fabric. *Textile& Clothing Sustainability*, *1*, 7.

Umaru, I. J., Badruddin, F. A., & Umaru, H. A. (2019). Phytochemical Screening of Essential Oils and Antibacterial Activity and Antioxidant Properties of Barringtonia asiatica (L) Leaf Extract. *Biochemistry Research International, 2019,*, 1-6.

VanderPorten, E. C. M. D., Scholle, J., Sherrill, J. C. Tran, Y., & Liu (2017). Identification of small molecule noncovalent binders utilizing SAMDI technology. *SLAS Discov*, *22*, 1211–1217.

Venkateshvaran, D., Nikolka, M., Sadhanala, A., Lemaur, V., Zelazny, M., Kepa, M., Hurhangee, M., A., Kronemeijer, J., Pecunia, V. and Nasrallah I., & *et al*., (2014). Approaching disorder-free transport in high-mobility conjugated polymers, *Nature, 515,530*, 384-388.

Vlajković, Jelena & Andrić, Filip & Ristivojevic, Petar & Radoičić, Aleksandra & Tesic,Zivoslav & Milojković-Opsenica, Dušanka. (2013). Development and validation of TLC method for the analysis of synthetic food-stuff dyes. *Journal of Liquid chromatography &amp Related Technologies*, *36*, 2476-2488.

Vulić, J., Čanadanović-Brunet, J., Ćetković, G., Tumbas, V., Djilas, S., Četojević-Simin, D., Čanadanović, V. (2012). Antioxidant and cell growth activities of beet root pomace ex-tracts. *J. Funct. Foods*, *4*, 670–678.

Vulic, J. J., Cebovic, T. N., Canadanovic-Brunet, J. M., Cetkovic, G. S., Canadanovic, V. M., Djilas, S. M., & Šaponjac, V. T. T. (2014). In vivo and in vitro antioxidant effects of beetroot pomace extracts. *Journal of Functional Foods*, *6*, 168-175.

Wang, X., Zhang, A., Yan, G., Han, Y., & Sun, H. (2014). UHPLC-MS for the analytical characterization of traditional Chinese medicines. *TrAC Trends in Analytical Chemistry*, *63*, 180-187.

Wangatia, L. M, Tadesse, K., & Moyo, S. (2015). Mango Bark Mordant for Dyeing Cotton with Natural Dye: Fully eco-friendly Natural Dyeing. *International Journal of Textile Science, 4*(2), 36- 41.

Warkkoyo, E. A. S. (2011). The solvent effectiveness on extraction process of seaweed pigment. *Makara Teknologi*, *15*, 5–8.

Windler, L., Height, M., & Nowack, B. (2013). Comparative evaluation of antimicrobials for textile applications. *Environment International*, 53, pp. 62–73.

Wolf, S., Schmidt, S., Müller-Hannemann, M., & Neumann, S. (2010). In silico fragmentation for computer assisted identification of metabolite mass spectra. *BMC Bioinformatics, 11* (1) 148.

Wruss, J., Waldenberger, G., Huemer, S., Uygun, P., Lanzerstorfer, P., Müller, U., & t al. (2015). Compositional characteristics of commercial beetroot products and beetroot juice prepared from seven beetroot varieties grown in Upper Austria. *J Food Compos Anal*., *42*, 46–55.

Xu, Y. *et al*. (2019). Structure, heterogeneity and developability assessment of therapeutic antibodies, *11*, 239–264.

Yamjala, K., Nainar, M. S., & Ramisetti, N. R. (2016). Methods for the analysis of azo dyes employed in food industry–a review. *Food chemistry, 192*, 813-824.

Yashwant, K. (2015). Beetroot: A Super Food. *International Journal of Engineering Studies and Technical Approach, 1,* 20-26.

Yim, H. S., Chye, F.Y., Rao V., Low, J.Y., Matanjun, P., How S. E, & Ho, C. W. (2013). Optimization of Extraction Time and Temperature on Antioxidant Activity of Schizophyllum commune aqueous extract Using Respose Surface Methodology. *J. Food Sci. Technol., 50*(2), 275-283.

Yusuf, M., & Mohammad, F. (2017). Natural colorant from Lawsonia Inermis leaves: Reflectance spectroscopy-induced optimal conditions of extraction and dyeing, in: *Plant-Based Natural Products: Derivatives and Applications,* S-U. Islam (Ed.), (pp.89–101).

Yusuf, M., Shabbir, M., & Mohammad, F., (2017). Natural colorants: Historical, Processing and Sustainable Prospects. *Nat. Prod. Bioprospecingt, 7*(1), 123–145.

Yusuf, M., Mohammad, F., Shabbir, M., & Khan, M. A. (2016). Eco-dyeing of wool with *Rubia cordifolia* root extract: Assessment of the effect of *Acacia catechu* as biomordant on color and fastness properties. *Textile and Clothing Sustainability*, *2*(10), 1–9.

Yusuf, M., Shahid, M., Khan, M. I., Khan, S. A., Khan, M. A., & Mohammad, F. (2015). Dyeing studies with henna and madder: a research on effect of tin (II) chloride mordant. *Journal of Saudi Chemical Society*, *19*, 64–72.

Zeng, S., Lu Wang, L., Teng Chena, T., Yuefei Wang, Y., Moc H., & Qua H. (2012). Direct analysis in real time mass spectrometry and multivariate data analysis: A novel approach to rapid identification of analytical markers for quality control of traditional Chinese medicine preparation. *Analytica Chimica Acta*, *733*, 38– 47.

Zheng, GH, Fu HB, & Liu G. P. (2011). Application of rare earth as mordant for the dyeing of ramie fabrics with natural dyes. *Korean J Chem Eng*, *28*(11), 2148–2155.

Zhou, H., Wu, L., Gao, Y., & Ma, T. (2011). Dye-sensitized solar cells using 20 natural dyes as sensitizers. *Journal of Photochemistry and Photobiology A: Chemistry, 219,* 188–194.

Zohary, D., Hopf, M., & Wesis, E. (2012). Domestication of plants in the old world: The origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin. *Oxford University Presson Demand*.

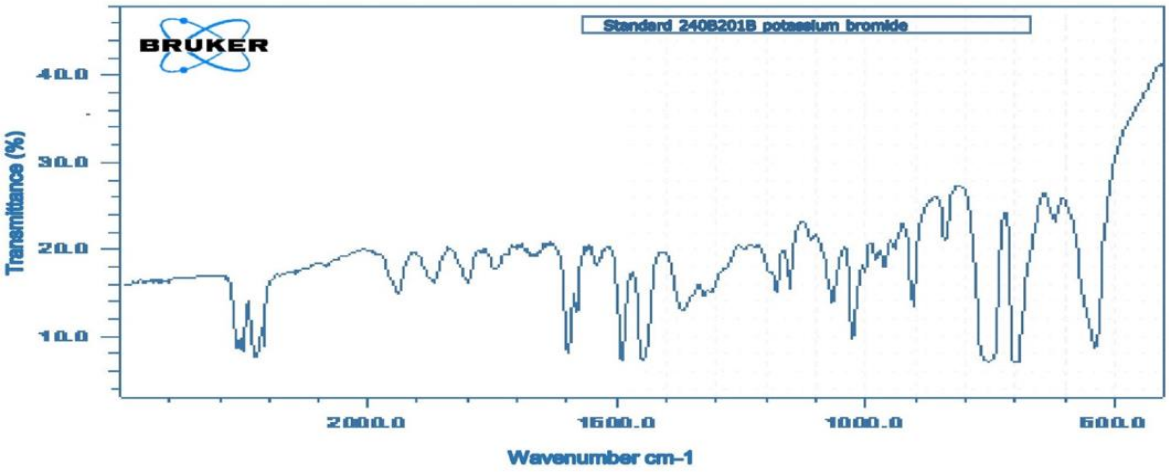
APPENDICES

Appendix 1: Dried and ground samples of *B. vulgaris* peels and pomace.

Appendix 2: Soxhlet apparatus in solvent extraction of *B. vulgaris* peels and pomace and crude extract.

Appendix 3: Serial dilution of the extract for UV-Vis analysis.

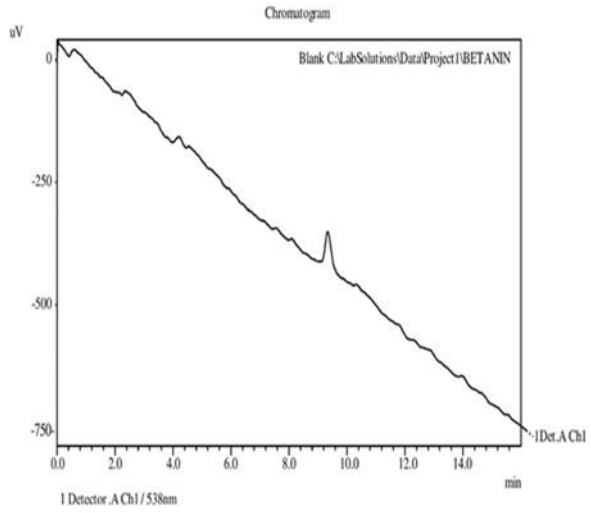
Appendix 4: FTIR spectrum of the standard (KBr -FTIR standard).



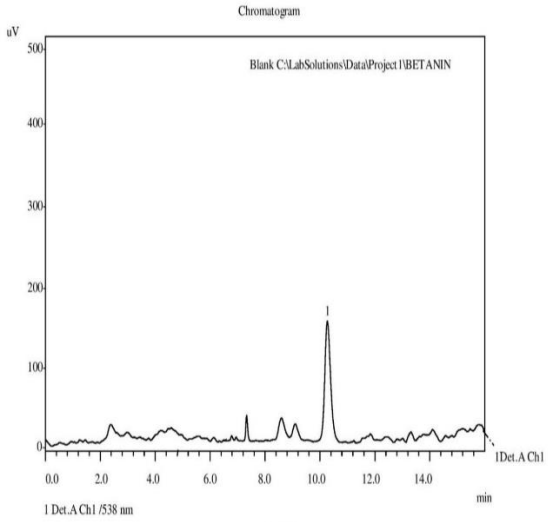
Appendix 5: Phytochemical screening of the *B. vulgaris* peels and pomace extract methanolic plant extract.

Appendix 6. HPLC chromatograms for solvent blanking (a) and (b) before and after standard injections respectively.

a

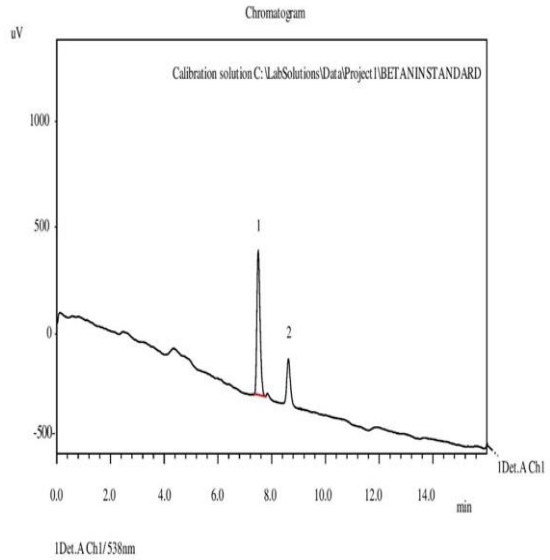


b

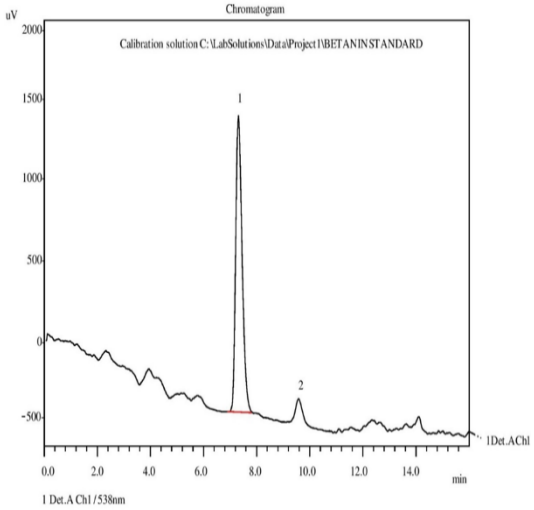


Appendix 7. HPLC chromatograms for betanidin 5-β-D-glucopyranoside standard at 10ppm (c) and 15ppm (d).

c

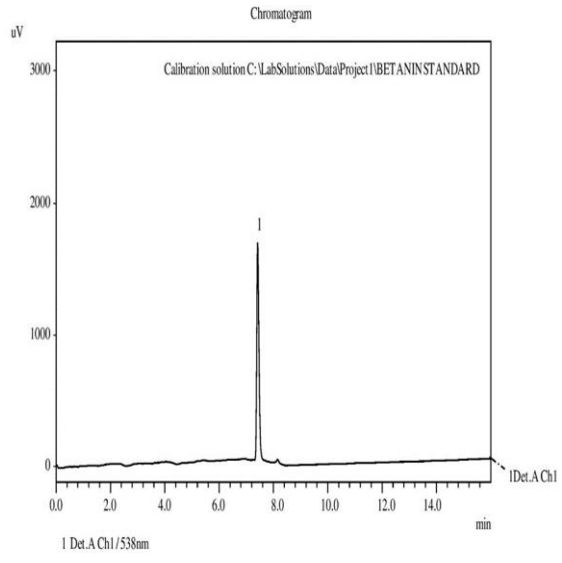


d

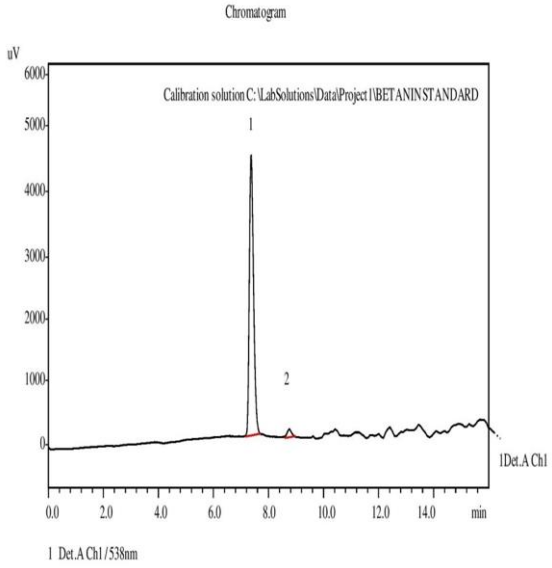


Appendix 8. HPLC chromatograms for betanidin 5-β-D-glucopyranoside standard at 20ppm (e) and 27ppm (f).

e

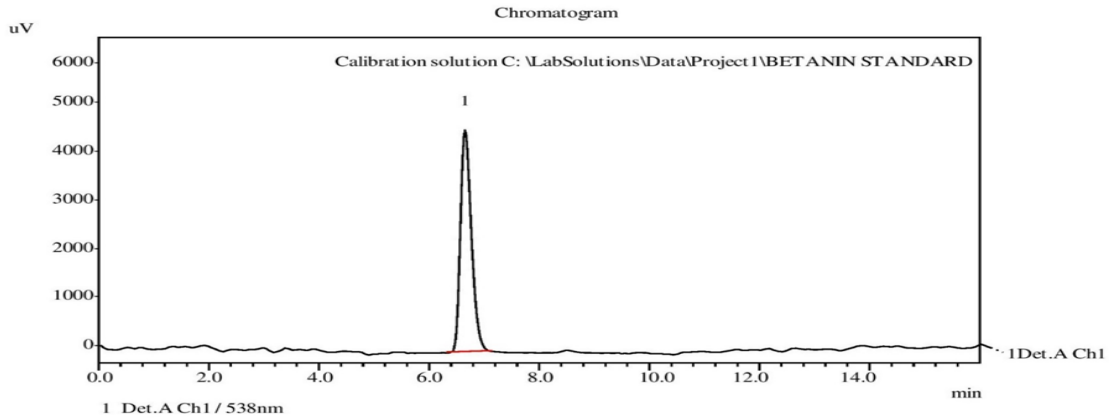


f



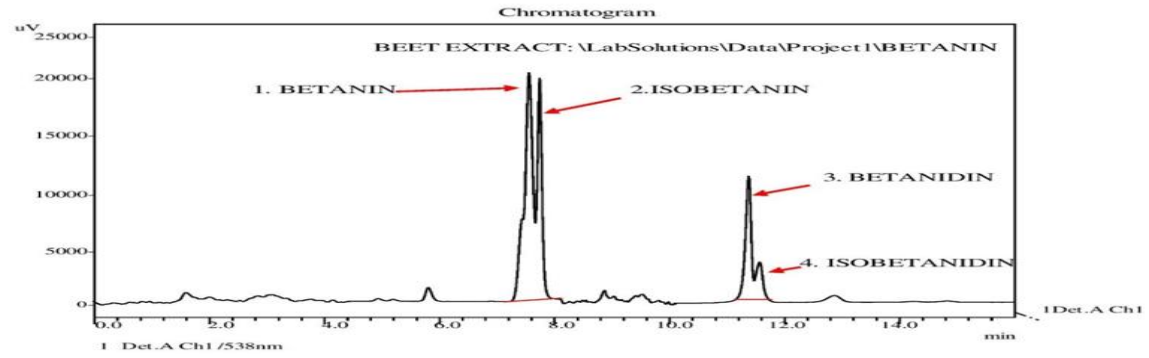
Appendix 9. HPLC chromatograms for betanidin 5-β-D-glucopyranoside standard at 35ppm (g).

g

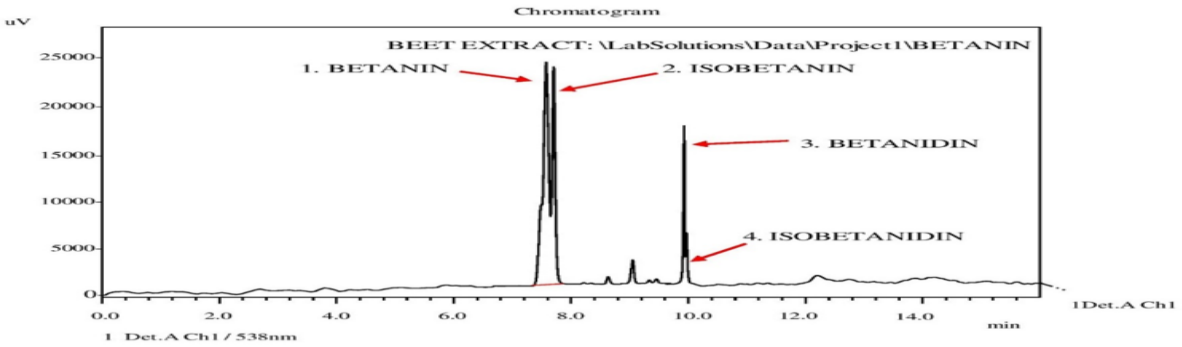


Appendix 10. HPLC chromatograms for the fractionated and purified sample triplicate injections; (h), (i) and (j) for first, second and third injections respectively, with UV detector at 538 nm.

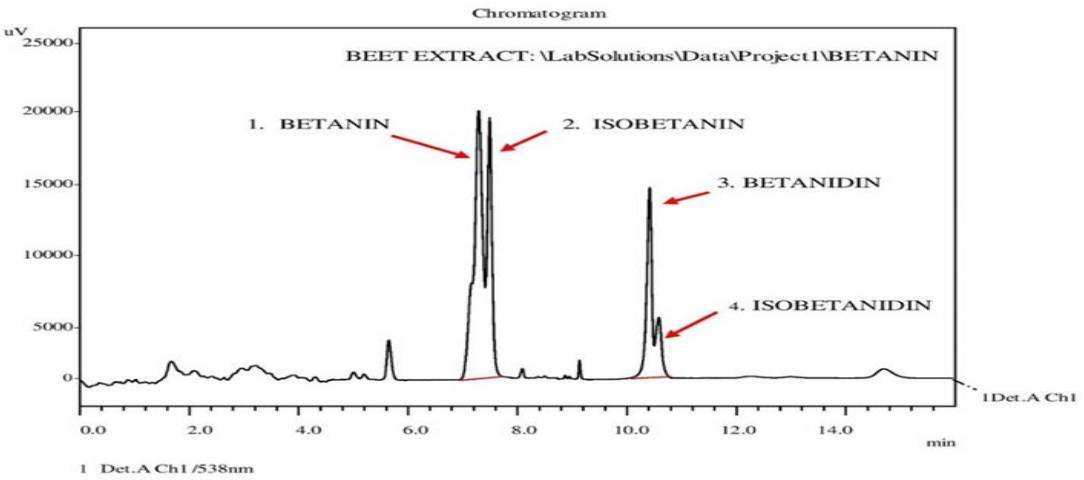
h



i

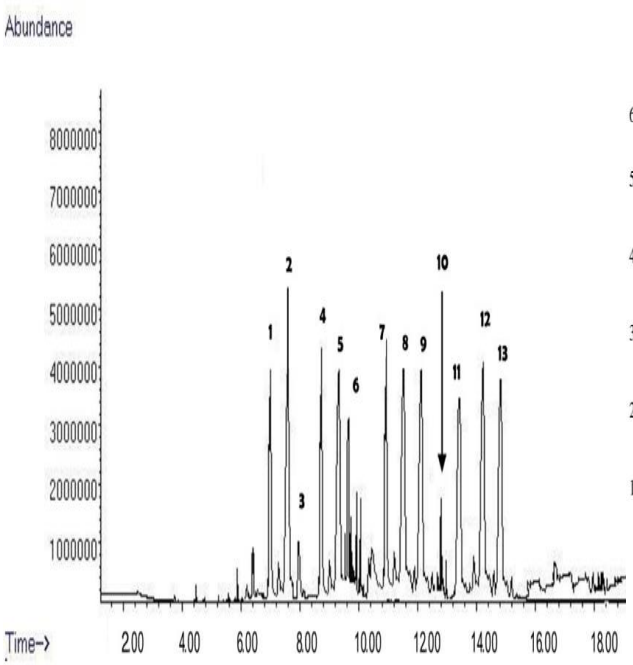


j

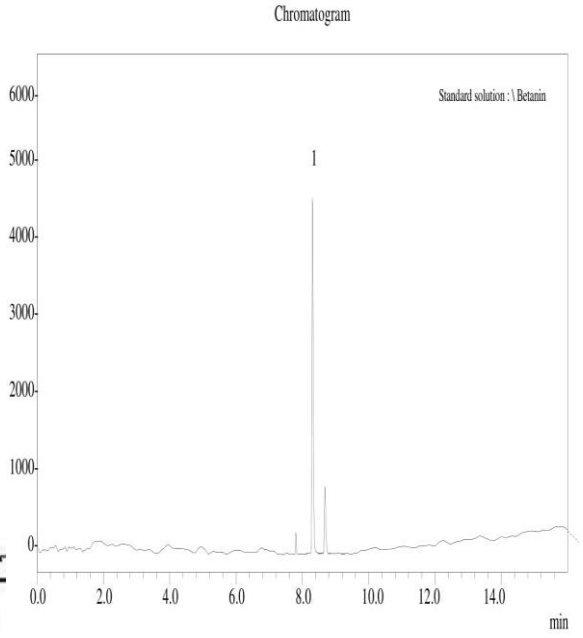


Appendix 11. The UHPLC-MS chromatograms; (a) for *B. vulgaris* peels and pomace pure sample, (b) for betanin standard and (c) mass spectrum of betanin compound in the sample, monitored at 538 nm. Peak numbers refer to the betalains listed in Table 4.13.

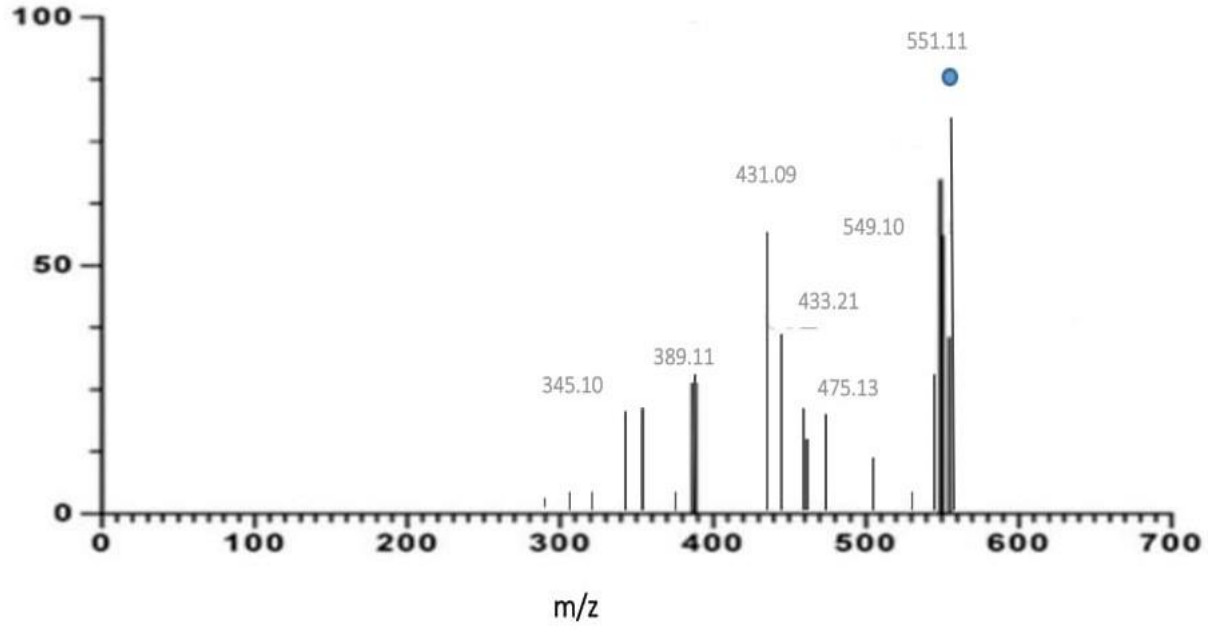
a



b



c



Appendix 12: Pictures of raw and dyed cotton fabrics dyed under different mordants, methods and conditions

Dyed fabric without mordant

Copper (II) Sulphate mordanted

Iron (II) Sulphate mordanted

P. dichromate mordanted

Tannic acid-alum mordanted

Reactive Orange HER