A STUDY ON EFFECT OF FROZEN STORAGE, LIGHT AND HEATING TEMPERATURE ON STABILITY OF ANTHOCYANIN OF NATURAL COLOR EXTRACTED FROM POMEGRANATE (*PUNICA GRANATUM* L.) PEEL

Farhana Sultana

Roll No: 0117/01

Registration No: 0448

Session: January-June/2017

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Food Processing and Engineering

Department of Food Processing and Engineering
Faculty of Food Science and Technology

Chittagong Veterinary and Animal Sciences University

Chittagong-4225, Bangladesh

December 2018

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.

(Mrs. Shireen Akther)

Supervisor

(Abdul Matin)

Chairman of the Examination Committee

Department of Food Processing and Engineering,

Faculty of Food Science and Technology

Chittagong Veterinary and Animal Sciences University

Khulshi, Chittagong-4225, Bangladesh

December 2018

Dedicated

To

My Beloved Family

Acknowledgements

All praises are due to the "**Almighty Allah"** for blessing me with the strength, aptitude and patience and enabled me to pursue higher education and to complete the thesis for the degree of Masters of Science (MS) in Food Processing And Engineering under the Dept. of Food Processing And Engineering, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh.

I pay heartily gratitude to **Professor Dr. Goutam Buddha Das**, Vice-Chancellor, Chittagong Veterinary and Animal Sciences University (CVASU) for giving special opportunity and providing such research facilities.

I have immense pleasure to express my deep sense of gratitude and indebtedness to my major advisor and research supervisor **Mrs. Shireen Akther** (Assisstant professor, Department of Food Processing and Engineering, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh) for her guidance and moral support. Her constant support, patience, valuable suggestions, useful criticism, constant encouragement and inspiration enabled me to overcome all hardship throughout this research and in preparation of the thesis.

I am highly grateful to **Abdul Matin (**Assistant Professor and Head, Department of Food Processing and Engineering, Chittagong Veterinary and Animal Sciences University, Chittagong) for his worthy inspiration and generous help in carrying out of the research work.

I feel proud in expressing my regard and immense indebtedness to Professor **Dr. Jannatara Khatun**, Dean, Faculty of Food Science and Technology and my respected all teachers for their kind co-operation, valuable suggestions and constructive criticism throughout the research period and for the thesis work.

I am also grateful to **Mrs**. **Musammet Rasheda Begum** (Associate professor, Dept. Of Agriculture and Social sciences) for her scholastic support for my research work.

I would like to thank both **Mohammad Mozibul Haque (Jewel)** sir (Lecturer, dept. Of appplied Food Scienc & Nutrition) and **Nazmul Sarwar** sir (Lecturer, Dept.Of Food Processing and Engineering) for their cordial support in my analytical work.

I would like to express my gratitude and cordial thanks to **National Science and Technology (NST)** Fellowship 2017-2018 of Ministry of Science and Technology,

Bangladesh and **Research and Extension** of Chittagong Veterinary and Animal Sciences University, Bangladesh for the financial help supplies during the study period. I would like to express cordial thanks to my loving friends and well-wishers for their cooperation, cheerfulness and inspiration during the course of this study. I also expresses my heartfelt respects and thanks to my beloved parents and brothers for their understanding, inspirations, moral support, kindness and blessings, forbearance and endless love to complete this study.

The author

December, 2018

Table of Contents

List of Abbreviation

Abbreviation	Elaboration
%	Percentage
Abs.S	The absorbance of the standard
Abs.T	The absorbance of the Test Sample
B	Blank
S	Standard
T	Test
°C	Degree Celsius
°F	Degree Fahrenheit
µG	Micro gram
µL	Micro litre
ADI	Acceptable daily intake
ANOVA	Analysis of vaiance
Aw	Water Activity
BC	Before Christ
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Ca	Calcium
CAC	Codex Alimentarius Commission
$CaCO_3$	Calcium carbonate
CC	Column chromatography
CE	Capillary electrophoresis
conc	Concentration
CVASU	Chittagong Veterinary and Animal Sciences University
CZE	Capillary zone electrophoresis
DF	Dilution factor
dL	Deci litre
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E110	Sunset yellow FCF
E122	Carmoisine/azorubine
EFSA	The European Food Safety Authority
ESI-MS	Electrospray ionization mass spectrometry technique
FAB-MS	Fast atom bombardment mass spectrometry
FD&C	Food, Drug, and Cosmetic Act
FDA	Food and Drug Administration
Fe	Iron
g	Gram
g/kg	Gram per kilogram
H/h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPCPC	High performance centrifugal partition chromatography
HPLC	High performance liquid chromatography

HPLC-MS	High-performance liquid chromatography-mass spectrometry
HSCCC	High-speed counter current chromatography
IC_{50}	Inhibitory concentrations
K	Potassium
KOH	Potassium hydroxide
kg-1	Per kilogram
l	Litre
LDL	Low density lipoproteins
Mg	Magnessium
mg	Miligram
min	Minute
mL	Mililitre
mm	Milimeter
mol/l	Mol per litre
ng	Nano gram
nm	Nanometer
NMR	Nuclear magnetic resonance
NS	Not significant
P	Phosphorus
PoMx	Pomegranate extracts
PoP	Pomegranate peel
PoPx	Pomegranate peel extract
ppm	Parts-per-million
PPO	Polyphenol oxidase
R.T	Room temperature
ROS	Reactive oxygen species
SA	Sample of jelly with artificial color
SC	Commercial sample of jelly
SN	Sample of jelly with natural color,
SO_2	Sulphur di oxide
SD	Standard deviation
$t_{1/2}$	Half-life
TAC	Total anthocyanin content
Temp	Temperature
TLC	Thin layer chromatography
TMCT	Tukey's Multiple Comparison Test
Trolox	6-hydroxy-2,5,7,8-tetramethychromane-2-carboxylic acid
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
w/w	Weight by weight
WR	Working Reagent

List of Figures

List of Tables

Abstract

Color is an important element in enhancing foodstuffs, constituting one of the major dietary additives. The anthocyanin pigment was extracted from Pomegranate (*Punica granatum* L.) peel, using Ethanol (99.99%). The extracted anthocyanin pigments then were exposed to number of environmental conditions, which could destabilize the anthocyanin molecules. These environmental conditions were included frozen condition (at -10^0C for three days intervals), various temperatures (30^0C, 45^0C, 60^0C and 75^0C) and presence or absence of light. The results showed that the stability of anthocyanins extracts have been significantly affected by frozen condition (62.96%), temperature (29.59%-51.61% degradation) and exposure to light (44.87%). The mineral content (Mg, Ca, P ,Fe and K) of natural (extracted from Pomegranate peel) and artificial color (mixture of E110, E122 and E330) analyzed by Humalyzer (3000) were 7.2 mg/100g, 20.6 mg/100g, 2.15 mg/100g, 1.4 mg/100g and 147.6 mg/100g respectively for natural color as well as 0.2 mg/100g, 0 mg/100g (no phosphorus), 0.39 mg/100g, 0.56 mg/100g, 7.2 mg/100g for artificial color respectively.The antioxidant capacity of natural color was done by DPPH method resulted in 0.04 µg/ml of IC_{50} value. The sensory analysis of the products as jelly with the addition of natural and artificial color and commercial was done based on hedonic rating test. There was no significant difference ($p<0.05$) in terms of the color, appearance and general acceptance of the samples as jelly.

Keyword: Color, anthocyanin, Pomegranate peel, antioxidant capacity, DPPH.

Chapter 1: Introduction

Color is an important quality attribute of foods. The objective of adding color to foods is to make them appealing, augment the loss of color during processing, to improve the quality and also to influence the consumer to buy a product. Color is added to food for the following reasons: a) To replace and restore color lost during processing, b) To enhance color that is already present, c)To minimize batch variations in processing and d)To color the uncolored food. Food colors can be grouped divided into four categories: a) Natural colors, b) Nature-identical colors, c) Synthetic colors and d) Inorganic colors (Naidu and Sowbhagya, 2012; Sahar and Manal 2012).

People associate certain colors with certain flavors (Delwiche, 2004) and the color of food can influence it perceived flavor. In fact, the color of a food influences not only the perception of flavor, but also attraction and quality and subsequently, consumption (Abd El-Galeel, 2002). Food manufacturers therefore often add colorings to their products to simulate or enhance a color that is perceived by the consumer as natural, to mask natural variations in food colors, to offset color loss due to light, extremes of temperature, moisture and storage conditions. In addition food colorants provide identity to foods, by color impartation on foods which would otherwise be colorless. And sometimes they are added just for effect or decorations purposes (Henry, 1996; Food Advisory Committee, 1987; Abd El-Galeel, 2002).

Food colorants are either natural or synthetic depending on source. Natural colorants are extracted from renewable sources such as plant materials, insects, algae, etc, while the synthetic colorants are manufactured chemically and are the most commonly used dyes in the food, pharmaceutical and cosmetic industries. The safety of synthetic colorants has previously been questioned, leading to a reduction in the number of permitted colorants. Due to this limitation and worldwide tendency towards the consumption of natural products, the interest in natural colorants has increased significantly (Huck and Wilkes, 1996). Of special interest to the food industry is the limited availability of red pigments (Hallagan, 1991; Lauro and Francis, 2000), therefore research into natural sources of red pigments have increased recently.

The limitations to the use of natural food colorants are their low stability in the food processing procedures, formulation and storage conditions and the fact that they may also impart undesirable odour or flavour to the food. The successful incorporation of

red cabbage and radish color components in food has been made possible only after the removal or substantial reduction of their strong aroma and flavour components (Giusti and Wrolstad, 2013). The selection of a food colorant is usually made after it is deemed satisfactory according to the following criteria: target shade, the physical/chemical attributes of the food matrix, stability to processing and storage conditions and regulatory issues (Giusti and Wrolstad, 2003).

Anthocyanins (Greek anthos, flower and Greek kyanose, blue) are the largest group of water-soluble pigments in the plant kingdom and belong to the family of compounds known as flavonoids which are part of an even larger group of compounds known as polyphenols (Mazza, 2007).

Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salt. Concentrations and types of anthocyanins in fruits vary significantly. Some factors, such as the types and cultivars of fruits (food matrix), as well as growth conditions, weather at the growing site, maturity, material preparation, and analysis methods could create differences in total anthocyanins in fruits. Growth conditions and environmental stress, such as high exposure to UV light and temperature, are important factors influencing the levels of bioactive content as a result of plant defense response (Leong and Oey, 2012). Maturity level also significantly contributes to the total anthocyanin content of fruits. The anthocyanin content in fruits is at its highest during the ripening stage, in which the biosynthesis rate is accelerated due to the action of the ripening hormone (ethylene), triggering the activation of many enzymes involved in anthocyanin biosynthesis, and eventually declining at the end of maturation stage. Since anthocyanins are synthesized at an increasing rate during maturation, the total anthocyanin content quantified here may serve as the index of maturity and an important quality parameter. The level of total anthocyanins also depends upon the cultivar. De Ancos et al. (2000) observed that frozen storage affects total anthocyanin content and individual anthocyanin distribution in different ways, depending on the cultivar. The anthocyanins are significant because of their nutraceutical benefits, antioxidant, and anticarcinogenic properties. Anthocyanins can also be used as natural food colorants in the food industry. However, anthocyanins are labile in nature, and therefore are susceptible to deterioration during processing and storage (Syamaladevi et al., 2011). Freezing is one of the most common methods of preservation of fruits for long-term storage. Frozen fruits are used as ingredients in

many food formulations such as jams, jellies, sauces, purees, toppings, syrups, juice concentrates, as well as bakery and dairy products. The freezing process and frozen storage may change the anthocyanin content of fruits, thereby affecting the antioxidant capacity and possible health benefits of the fruit. Therefore, it is vital to understand the stability of anthocyanins during frozen storage. For frozen fruits, the retention of anthocyanins depends on the freezing rate, composition, pH, cultivar, temperature, and the presence/absence of oxygen (Wrolstad et al., 1970; Mazza and Miniati, 1993).

The major problem of anthocyanins use as natural food colorants is their instability, either in simple or in complex food formulations. Anthocyanins are stable under acidic conditions (pH 2), but under normal processing and storage conditions they transform to colorless compounds and subsequently to insoluble brown pigments. A number of factors influence the stability of anthocyanins, such as temperature, pH, light, oxygen, enzymes, presence of ascorbic acid, sugars, sulphite salts, metal ions and copigments (Francis, 1989; Jackman, Yada, Tung, & Speers, 1987).

Pomegranate *(Punica granatum* L. Punicaceae; the common name is derived from Latin words ponus and granatus), a seeded or granular apple, is a delicious fruit consumed worldwide. The fruit is native to Afghanistan, Iran, China and the Indian subcontinent. The ancient sources of pomegranate linked Iran to Pakistan, China and eastern India, where pomegranates had been under cultivation for thousands of years. From the west of Persia (modern day Iran), pomegranate cultivation stretched through the Mediterranean region to the Turkish European borders and American southwest, California and Mexico (Celik et al., 2009; Lansky and Newman, 2007).

Pomegranate can be divided into three parts: Seeds, peel, and juice, all of which seem to have medicinal benefits. Pomegranate extracts have been used since ancient times to treat several conditions including parasitic and microbial infections, diarrhea, ulcers, aphthae, hemorrhage, and respiratory complications (Naqvi et al., 1991 and Viladomiu et al., 2013).

The pomegranate peels make up about 60% of the fruit, and they are rich in many compounds such as phenolics, flavonoids, ellagitannins (including punicalagins), proanthocyanidin compounds, complex polysaccharides, and many minerals(Viuda-Martos et al., 2010). The peel is the part of the fruit with the highest antioxidant activity, which is in line with its high content of polyphenols. The results of investigation and

comparison of antioxidants of the peel, pulp and seed in 28 different spices show that pomegranate peel has the highest antioxidant activity (Guo et al., 2003 and Li et al., 2006). Anthocyanins, an antioxidant in peel, are the most important and abundant natural pigments belonging flavonoid family (Negi et. al., 2003 and Williams and Grayer, 2004). These compounds are concentrated in pomegranate peel (PoP) and juice, which account for 92% of the antioxidant activity associated with the fruit (Afaq et al., 2005; Negi et al., 2003; Zahin et al., 2010).

For centuries, various parts of the plant have been used for medicinal purposes. Practitioners of Ayurvedic and Unani system of medicine have used pomegranate as a therapeutic agent for the treatment of inflammatory diseases and disorders of the digestive tract (Seeram et al., 2005; Lansky & Newman, 2007). Its juice, peel and oil have also been reported to have anticancer and cardiovascular preventing properties (Kim et al., 2002). Pomegranate fruit arils are also very popular due to their taste. The arils are processed to delicately flavored juice, squash, jelly, jam, wine, anardhana, etc. Due to the rich color, sweet-sour flavor and high antioxidant content, manufacturers tend to add pomegranate to products such as jelly, ice creams, truffles and chewing gum. A huge amount of pomegranate peel waste is thus produced, disposal of which has become an environmental problem. It has been reported that the peel and seed fractions of some fruits have higher bioactivities than the pulp fractions (Tomas-Barberan & Espin, 2001; Guo et al., 2003; Balasundram et al., 2006). There are reports on the antioxidant potential of Pomegranate peel extract (PE) (Negi & Jayaprakasha, 2003; Ricci et al., 2006; Yasoubi et al., 2007).

The therapeutic potential of PoP has been widely recognized by different cultures. In Egyptian culture, several common ailments such as inflammation, diarrhea, intestinal worms, cough and infertility have been treated by exploiting pomegranate peel extract (PoPx). The exceptional antioxidant potential and strong medicinal properties of PoP led the international scientific community to initiate intensive research in the last decade to further investigate its role in human health (Lansky and Newman, 2007).

Several studies have demonstrated the antimicrobial, antihelminthic, and antioxidant potential of the active ingredients of pomegranate extracts (PoMx), suggesting their preventive and curative role in gastro-mucosal injuries, cancer chemoprevention, ethanol- and acetone-induced ulceration and diabetic oxidative damage (Al-zoreky and Nakahara, 2003; Arun and Singh, 2012; Negi et al., 2003).

1.1 Rationale and Significance of this Study:

The rationale of doing this study is pomegranate are available in Bangladesh. The peels of pomegranate are usually thrown away after eating it's arils. Rather than wasting them, they can be useful as a natural source of natural colorants.

The significance of doing this study is to investigate the best condition to extract colorants from pomegranate peels in order to make it last long. If the colorants can last long, it can be used for a longer period. Besides, this study is done to determine the intensity of colorants exist in pomegranate peels. The optimum anthocyanins in a sample will be chosen to be the best sample and can be used as colorants in food production. The outcomes from this study can also open up opportunities to increase the demand of natural colorants for food industry in Bangladesh.

1.2 Aim and specific objectives of the study:

1.2.1Aim of the study:

The overall aim of the study is to assess the effects of frozen storage, light and heating temperature on the stability of anthocyanin of natural color extracted from pomegranate peel.

1.2.2 Objectives:

i. To study the storage and heat stability of the extracted natural food colors.
ii. To assess the antioxidant activity of natural color extracted from pomegranate peel.
iii. To compare the minerals (Mg, P, Ca, Fe, K) between natural and artificial colors.
iv. To assess the sensory quality characters of the developed food products (jellies) using natural and artificial color.

Chapter 2: Riview of literature:

2.1 Color and colorants in foods:

Color is a visual feature that arises from the spectral distribution of the light. The formation of light occurs from the interaction of matter and light, and humans see wavelengths between 380-770 nm. As for the other matters in nature, the colors of the food are also based on this basic principle (Demirağ and Uysal, 2006).

Color that affects taste recognition and product acceptability might has influence on both actual and perceived nutritional value of food (Branen and Haggaerty, 2001).

Color additives or colorants are any dye, pigment or substance that are capable of coloring (either alone or by reacting with other substances) when added to food, medicament, cosmetics, or applied to the human body (FDA, 2010).

Today, due to the development of the food industry, the need to colorize food products has increased for various reasons. The Codex Alimentarius Commission (CAC) defines colorants as substances added to color food or to correct the color of the food. In addition, the colorants are added to restore the natural color lost during processing and storage of the food, to enhance the existing color, to strengthen the weak color, to color the food which is actually colorless and to win consumers by hiding low quality (Demirağ and Uysal, 2006; Aberoumand, 2011; Pandey and Upadhyay, 2012).

2.1.1 Classification of colorants:

Directives on colorants are examined in 3 groups. These are as follows:

1. Colorants whose ADI values are determined and allowed for use,
2. Colorants permitted to use only for special purposes (such as surface finishing) ($CaCO_3$, aluminum, silver, gold),
3. Colors that are only allowed to use in some foods (Titanium dioxide, vegetable carbon, red beet)

Colorants differ from each other by various properties such as chemical structures, sources, and purpose of use. As it is difficult to classify the colorants according to these properties, they are divided into two groups based on their sources as natural and synthetic (Demirağ and Uysal, 2006).

2.1.2 Booster for Natural colors:

i. As there is increasing awareness about the harmful effects of usage of synthetic colors and the chemicals obviously demand for natural food colors in the international market abruptly increases.

ii. As Japan and all European countries have banned trading of synthetic color made products.

iii. As encouragement for using Natural food colors in novel products like infant toys and crayons, organic textile printing, handmade paper etc has been implemented and followed in few developed countries.

But colors from plant, animal and mineral sources also sometimes called as biocolors, which were used in earlier times, had their own drawbacks like heat, pH and light instability, and against oxidizing agents in food, which made synthetic colors gain popularity in food industry. In contrast, chemically synthesized colors were easier to produce, cinexpensive, and superior in coloring properties as they blend easily. As the use of synthetic colors in food increased, the safety concerns are also raised through numerous regulations across the world and in the USA, only seven synthetic colors are permitted. In India according to The Prevention of Food Adulteration Act of India the use of eight synthetic colors in specified food commodities at a uniform level of 100 mg kg-1 or mg l-1 is permitted. (Magoulas, 2009 and Fiszman et. al., 2012).

2.1.3 Sources of Natural Dyestuffs:

Plant sources include roots, berries, flowers, barks and leaves. Red color (dyer's root from Madder plant, Brazilwood, beetroot, cranberry, safflower and orchil), orange color (stigmas of saffron flower), yellow color from (Camomile and Milkwort flowers and Weld), green color (ripe Buckthron berries, ragweed) and blue color (Woad plant and Spirulina). The most important dyes extracted from animal sources are Natural Sepia (from the ink sac of the cuttlefish), Crimson (From the Kermes Louse) and Tyrian purple (from the Murex shellfisf). Common color and food associations are noted in the table 2**.** (Mahony A, 2001; Sahar and Manal, 2012)

Table-1 Common color and associated food (Lakshmi, 2014**)**

Sl.No	Color	Chromophore	Plant Sources	Nutrients
1	Purple-blue	Anthocyanins	Eggplant, blackberry, purple, cabbage, plum, blueberry, raisins, prunes, purple grapes, Fig.	Lutein, zeaxanthin, resveratrol, vitamin C, flavanoid, ellagic acid, quecertin
2	Green	Chlorophyll	Avocado, cucumber, spinach, kale, broccoli, snow pea, zucchini, artichoke, lettuce, kiwi	Lutein, zeaxanthin, vitamin C, calcium, folate, β-carotene
3	White-tan	Anthoxanthins	Cauliflower, mushrooms, parsnip, potato, ginger, onions, jicama, banana, garlic, onions	Ancillin, Potassium, Selenium
4	Yellow-orange	Carotenoids	Papaya, pineapple, apricot, pumkin, peach, peach, carrot, orange, corn	β-carotene, zeaxanthin, flavanoid, vitamin C, Potassium
5	Red	Lycopene or Anthocyanins	Cranberry, beet, water melon, tomato, strawberry, pomegranate	Ellagic acid, quecertin, Hesperidin etc.

2.1.4 Natural Food Colors:

The natural colors are extracted from a variety of sources such as seeds, fruits, bark, leaf, root, stem, wood flower, rhizome and whole plant by conventional methods. Being biological in origin, they are often called as 'bio colors' (Indu et al.2004). The natural color of foods may be the result of the presence of natural pigments such as carotenoids, chlorophylls, myoglobins and anthocyanins and chemical modification during the processing of natural constituents of foods, e.g. caramelization and color additives (Parkinson and Brown, 1981). A suitable natural color can be developed by manipulation of certain factors such as pH, heat, light, storage and other ingredients.

2.1.5 Plants Yielding Natural Dye/color:

Among the biological sources, many natural dyes/colors are obtained mainly from plants, producing various colors such as red, yellow, blue, black, brown and a combination of these. Green is the dominant pigment in plants, while the carotenoids

are a large group of pigments associated with chlorophyll and responsible for autumn leaf pigmentation. Many of the intense colors in flowers and fruits are contributed by the flavonoid pigments and closely related compounds with a diverse range of colors, which are the result of structural differences between the compound and the relative concentration of specific pigments within the cells. Betalains, a restricted group of pigments, get their name from the red–violet pigment isolated for the first time in crystalline form from the root of the beet *Beta vulgaris,* L (Kays, 1998). Natural colorants and pigments from plants have use in coloring food, beverages, soft drinks, confectionery, bakery products, etc. **(**Table:3). Colors of plant origin are environmentally friendly and non-toxic and hence preferred over synthetic colors. Over 2000 pigments have been reported to be produced by various parts of plant, of which only little more than 150 have been exploited commercially (Siva, 2007).

Table 2: Sources of natural food colors, pigments and their applications (Hirasa, 1998; Mittal et al., 2007)

Color	Source	Applications/uses
Chlorophylls/chlorophyllin		
Natural green and green	Nettles, grass, algae, alfalfa, celery, collard greens, sea vegetables, green beans, peas, green olives, parsley, spinach, green turnips, asparagus, bellpeppers, broccoli, Brussels sprouts, green cabbage, barley and other herbs.	Pastas, confectionery, medicines, processed food, vegetable oils, delicatessen, spice preparations, ice cream and coloring materials.
Carotenoids and xanthophylls		
Natural orange	Mushrooms	Tanningpills, fruitspreads, candies, syrups, sauces and carbonated drinks.
Red, dark red and purple red	Paprika and red pepper	Beverages, processed food and tomato products.
Flavonoids/anthocyanins		
Red, purple and blue	Strawberries, grapeskin, blackgrapes, raspberries	Confectionery, food products and dessert products.
Red	Redcabbage	Coloring chewing gum and vegetable juice, making drinks free from nasty smell and sedimentation.

2.1.6 Chemistry of Natural Food Colors:

2.1.6.1 Carmine (Natural Red 4):

Dactylopius coccus (Cochineal) is a local insect in South America and Mexico. The pigment obtained from this insect and its egg is carminic acid (Lakshmi, 2014). Carmine is a compound that carminic acid creates with aluminum pigment. Since it is an expensive substance as a color additive it is not economical to use in food industry.

2.1.6.2 Chlorophylls:

Chlorophyll is the green pigment utilized by all higher plants for photosynthesis. The name derives from the Greek words for green and leaf (compared to xanthophyll). Chlorophyll is a cyclic tetrapyrrole with coordinated magnesium in the center. In plants, there are two forms of chlorophyll (a and b) which only differ in the substitution of the tetrapyrrole ring. It is used in jam, jelly, candy, ice cream and in several other products, but chlorophyll finds limited use as a colorant because of the lability of the coordinated magnesium and the associated color change. Chlorophyll is extracted from edible plants, nettle, grass, or alfalfa, silkworm droppings and mulberry leaves (Mortensen, 2006).

2.1.6.3 Anthocyanin:

Anthocyanins form the attractive red to blue colors in many fruits, vegetables, flowers and leaves. They were glycosides of water called anthocynidins and are biogenetically closely related to the larger group of flavonoids. They were used for coloration of carbonated beverages, fruit drinks, jam, jellies etc., (Lewis et al., 1977) (Discussed in details in the section 2.2).

2.1.6.4 Carotenoids:

Carotenoids are a group of phytochemicals responsible for yellow, orange, red and violet colors of foods and having an important role in the prevention of human diseases and maintaining good health. In addition to being potent antioxidants some carotenoids also contribute to dietary vitamin A. Although the chemistry of carotenoids has been studied extensively, their bioavailability, metabolism and biological functions are only now beginning to be investigated (Rao and Rao, 2007).

2.1.6.5 Annatto:

Tropical annatto is a pigment derived from the pericarps of Bixa orellana L. tree seeds (Satyanarayana A et al., 2010). Having yellow-orange food colorant property, annatto is used in smoked fish, various beverages, bakery products, and dairy industry

(Solymosi, 2015). It has also been reported that annatto is used especially in cheese, butter, margarine and snacks (Giuliano et al., 2003).

2.1.6.6 Lycopene:

Lycopene is the pigment principally responsible for the characteristic deep red color of ripe tomato fruits and tomato products; it is also found in watermelon, papaya, pink grapefruit and pink guava. Processed tomato products are more available dietary sources of lycopene than fresh tomatoes. Lycopene is a member of the carotenoid family; it is a natural fat-soluble pigment found in certain plants and microorganisms, where it serves as an accessory light-gathering pigment and to protect these organisms against the toxic effects of oxygen and light (Rao and Agarwal, 1999).

2.1.6.7 Turmeric/curcumin:

The yellow color additive turmeric is the ground powder of the rhizomes of *C. longa* L. Turmeric contains 3–5% volatile oils and 2.5–6% curcuminoids. Turmeric oleoresin contains 15–40% curcuminoids, apart from volatile oils and other extractable plant constituents (Marmion, 1991). Turmeric has been attributed a number of medicinal properties. No convincing evidence of allergies to the spice has been so far reported.

2.1.6.8 Saffron:

Saffron color comes from the crocus plant, each blossom of which contains one pistil, consisting of three stigma, a style and an ovary. The saffron spice consists of the dried stigmas and style of the crocus bulb, the coloring matter of which is crocin. Crocin is readily hydrolysed to crocetin and D-glucose in vivo (Farrell, 1985).

2.1.7 Natural Color Additives and Regulations Regarding Their Use:

Today, in all countries of the world, food additives and especially colorant-related regulations are in focus. Moreover, despite global cooperation and harmonization efforts, these regulations vary from country to country (Lehto et al., 2017). The European Food Safety Authority **(**EFSA) and the Food and Drug Administration **(**FDA) are the most important regulatory bodies authorized to protect and improve human health, as well as to ensure the quality and safety of food products (Martins et al., 2016). The FDA has primary legal liability for determining and regulating the safe use of food additives.In developed countries, the use of colorants in the food industry depends on a number of toxicity tests (such as detection of the acute, subchronicand chronic toxicity, carcinogenicity, teratogenicity, reproductive toxicity, accumulation in the body, bioenergy effects and immune effects) (Amchova et al., 2015).

2.2 Anthocyanins:

Anthocyanins are natural colorants belonging to the flavonoid family of secondary metabolites; they are a diverse group of intensely colored pigments synthesized in plants and bacteria responsible for the often brilliant orange, red, purple and blue colors in fruits, vegetables, flowers, leaves, roots and other plant storage organs. They are found predominantly in outer cell layers such as the epidermis and peripheral mesophyll cells. They are watersoluble, facilitating their easy use in aqueous media as food colorants (Markakis, 1992) and have been used without any adverse effects for centuries.

In fact, more than 700 anthocyanin structures have been identified from plant extracts, and almost 200 different anthocyanins have been presented with tentative structures (Andersen and Jordheim, 2014). Among these, the most commonly found anthocyanins are based on six anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Fernandes et al., 2014).The distribution of the six most common anthocyanidins in fruits and vegetables is approximately as follows: cyanidin 50 %, delphinidin 12 %, pelargonidin 12 %, peonidin 12 %, petunidin 7 % and malvidin 7 % (Kong et al., 2003).

The predominant anthocyanins in fruits are cyanidin-3-glucoside, delphinidin-3-glucoside, malvidin-3-glucoside, pelargonidin-3-glucoside, and petunidin-3-glucoside (Dugo et al., 2003; Hillebrand et al., 2004).

The glycoside derivatives that more widespread in nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides. The presence of the 3-glucoside derivatives is about 2.5 more frequent than the 3,5-diglucosides and the most common anthocyanin is cyaniding-3-glucoside (Kong et al., 2003).

2.2.1 Function of Anthocyanins:

In plants, anthocyanins also act as photoprotectants by scavenging free radicals that are produced during photosynthesis (Hiemori et al., 2009). This function is attributed to their ability to donate protons to highly reactive free radicals (Castañeda-Ovando et al., 2009), which is conferred by the anthocyanins' structure—the number and organisation of the phenyl groups, the availability of electron-donating and electron-withdrawing groups in the ring structure, the degree of structural conjugation and the positive charge of anthocyanins.

In doing so, they prevent further radical generation and protect cells from oxidative damage which has often been associated with aging and various diseases caused by oxidative stress (Bueno et al., 2012). Studies have demonstrated that anthocyanins can help in the prevention of cardiovascular and neurological diseases, cancer and inflammation (Konczak and Zhang, 2004). They have also been shown to be effective in obesity and diabetes control, and in improving visual functions (Tsuda, 2012). Owing to their antioxidant capacity and the associated health benefits, anthocyanins have gained increased attention in recent years.

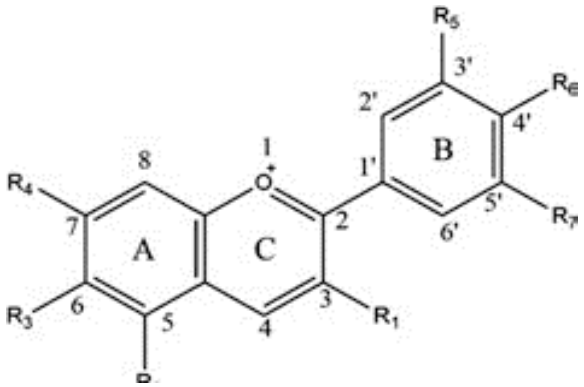

Figure 1: General structure of anthocyanin

2.2.2 Stability of Anthocyanins:

The stability of anthocyanins is dependent on several factors including the structure of the anthocyanins, temperature, pH, light intensity, oxygen availability and presence of enzyme, ascorbic acid, sugar and metal ion in the system (Roobha et al., 2011). Isolated anthocyanins are very unstable and highly susceptible to degradation (Giusti and Wrolstad, 2003).

2.2.2.1 Structure Influence:

The stability of anthocyanins is influenced by the ring B (Figure. 1) substituents and the presence of additional hydroxyl (–OH) or methoxyl ($–OCH_3$) groups which decrease the aglycon stability in neutral media; therefore, pelargonidin is the most stable anthocyanidin (Fleschhut et al., 2006).

Acylated anthocyanins are more stable than the corresponding non-acylated ones. Acylated anthocyanins appear to prolong their half-life and slow down their decaying process under thermal treatment compared to nonacylated derivatives, and a higher degree of hydroxylation in the B-ring would decrease their half-life (Sadilova et al.,

2006). Acylation of the molecule is believed to improve anthocyanin stability by protecting it from hydration (Goto et al. 1979; Raymond 1983).

2.2.2.2 Co-pigmentation Influence:

Co-pigmentation is a phenomenon in which the pigments and other colorless organic compounds, or metallic ions, form molecular or complex associations, generating a change or an increment in the color intensity (Boulton, 2001). Some investigations suggest that the co-pigmentation of anthocyanins with other compounds is the main mechanism of stabilization of color in plants (Davies and Mazza 1993; Mazza and Brouillard, 1990). The magnitude of the co-pigmentation effect is pH dependent. The co-pigment effect is evident under weakly acid conditions (pH 4–6) (Castañeda-Ovando et al., 2009).

2.2.2.3 P^H Influence:

Anthocyanins are found to be typically more stable in acidic media at low pH values than alkaline solutions with high pH values. Cabrita et al. (2000) showed that even though six common anthocyanin 3-glucosides (i.e. pelargonidin, peonidin, malvidin, cyanidin, petunidin and delphinidin) displayed the most intense red coloration and the highest stability in aqueous solutions of pH 1–3, however, the first three also exhibited intense and stable bluish colors at pH 8–9, highlighting the possibility of using them as colorants in alkaline food products.

2.2.2.4 Thermal Influence:

Temperature, in particular, has shown to be one of the major factors in anthocyanin degradation during food processing. The stability of anthocyanins in foods was found to decrease during processing and storage as temperature rises (Maccarone et al. 1985; Mercadante et al., 2008). Many studies have been carried out to investigate the thermal degradation of anthocyanins in roots of Chinese red radish (*Raphanus sativus L.*) (Jing et al., 2012), as well as the effects of temperature and cooking time on the stability of individual anthocyanins in black rice (Hou et al., 2013). Fischer et al. (2013) also reported that heating of pomegranate juice at 90 °C resulted in anthocyanin degradation, which caused discolorisation. Even at lower temperatures, degradation of anthocyanins is still a concern. Hellström et al. (2013) found that the half-life (**$t_{1/2}$**) of anthocyanins in both laboratory made and commercial berry juices was much shorter at room temperature than at 4 and 9 °C. During storage at 4 °C, Reque et al. (2014) found that

anthocyanins in blueberry whole juice were degraded, although its antioxidant capacity remained stable.

2.2.2.5 Solid Content Influence:

The degradation rates of anthocyanins increase with increasing solid content during heating. This could be due to closeness of reacting molecules in juice with higher soluble solid content (Patras et al., 2010). In strawberry, anthocyanin degradation occurs as soon as strawberries are processed into juice or concentrate and continues during storage. This degradation of anthocyanins is greater in concentrates compared to juices (Garzón and Wrolstad, 2002). Similar trends have been reported for anthocyanins in sour cherry (Cemeroglu et al., 1994).

2.2.2.6 Water Activity (Aw) Influence:

Water activity is another factor influencing the stability of anthocyanins. Reduced water activity enhances anthocyanin stability. Anthocyanin pigments in dried forms can exhibit remarkable stability. Anthocyanins are stable when stored in dry crystalline form or on dry paper chromatograms, which leads to the hypothesis that water is involved in discoloration reactions (Markaris et al., 1957). The degradation of anthocyanins in freeze-dried strawberry puree increases when stored at high relative humidity conditions (Erlandson and Wrolstad, 1972). A study also reported the same trend that increasing Aw increased the degradation rate of anthocyanins (Garzón and Wrolstad 2001).

2.2.2.7 Oxygen Influence:

Oxygen amplifies the impact of anthocyanin degradation processes (Cavalcanti et al., 2011). Specifically, this can take place through a direct oxidative mechanism or through the action of oxidising enzymes such as polyphenol oxidase (PPO). For example, PPO catalyses the oxidation of chlorogenic acid to chlorogenoquinone, which reacts with anthocyanins to form brown condensation products (Patras et al., 2010). It was reported that oxygen-restricted atmospheres best retained initial antioxidant capacity and anthocyanin content of fresh-cut strawberries over a period of cold storage (Odriozola-Serrano et al., 2009). Although Zheng et al. (2003) reported that oxygen from 60–100 % increased anthocyanin content of freshly harvested blueberries in the initial period of cold storage (0–7 days), they later reported that a similar effect in strawberries decreased with prolonged storage (Zheng et al., 2007).

2.2.2.8 Enzymatic Degradation:

There are a number of enzymes, endogenous in most plant tissues, which have been implicated in the decoloration of anthocyanins. Generally, these have been identified as either glycosidases (Huang 1955; Forsyth and Quensel 1957) or phenolases (Peng and Markakis 1963), although Grommeck and Markakis (1964) have also reported a peroxidase-catalyzed anthocyanin degradation.

Huang (1955, 1956a) investigated the decolorizing action of several crude fungal enzyme preparations on solutions of pure anthocyanin within a pH range of 3.0 to 4.5. Enzymatic decoloration was attributed to the action of glycosidases (anthocyanases) which hydrolyzed the anthocyanin to anthocyanidin and sugar, with the destabilized aglycone spontaneously degrading to colorless derivatives (Huang 1955, 1956a; Forsyth and Quesnel 1957). The transformation of liberated aglycone to colorless derivatives and the enzymatic deglycosylation of anthocyanins by fungal anthocyanase was found to follow first order kinetics at pH 3.95 and 30 ^{0}C (Huang 1956b).

A fungal anthocyanase preparation was used by Yang and Steele (1958) to remove excess anthocyanin from concentrated products (blackberry jams and jellies) where pigmentation was too dark and unattractive. Huang (1955) had suggested the use of such enzyme preparations in the manufacture of white wines from mature red grapes. Wagenklecht et al. (1960) attributed the loss of red color associated with scald in sour cherries to endogenous anthocyanase activity.

2.2.2.9 Ascorbic Acid:

Beattie et al. (1943) were among the first researchers to observe the concurrent disappearance of ascorbic acid and anthocyanin in stored fruit juices, and to suggest a possible interaction between the two compounds. Studies by Sondheimer and Kertesz (1953) suggested that maximal loss of anthocyanins occurred under conditions most favorable to ascorbic acid oxidation. This indicates the importance of oxidation products, rather than the ascorbic acid itself, in anthocyanin destruction. Although hydrogen peroxide is a product of ascorbic acid oxidation, its formation in strawberry juice has not been demonstrated. Despite this, Sondheimer and Kertesz (1952) demonstrated significant losses in color due to the presence of added H_2O_2 in strawberry juice.

Markakis et al. (1957) demonstrated a synergistic effect between ascorbic acid and oxygen on pigment degradation. Starr and Francis (1968) similarly observed that

maximum pigment losses in cranberry occurred when high levels or concentrations of oxygen and ascorbic acid were added to the juice.

2.2.2.10 Sulfur Dioxide:

Sulfur dioxide, commonly used as a preservative in the food industry, has been found to stabilize the anthocyanins of some berry fruits at low concentrations, and is evidently more efficient in combination with ascorbic acid or rutin, a copigment (Adams 1973a; Williams and Hrazdina 1979). Despite this stabilizing effect, however, the practical application of such a treatment is limited due to the decolorizing effect of sulfite on the pigments (acidification to about pH 1 is required for restoration of the red color) (Jurd 1964a).

2.2.3 Stabilization:

The preservation of anthocyanin coloration has always been a challenge to the food processing industry. As the number of "coal-tar dyes" permitted for use in food is gradually reduced, the need for better retention of the original pigments in the commodity becomes dire. Measures which may maximize the anthocyanin coloration of foods are listed below:

1. A plan for anthocyanin color preservation should begin with the procurement of intensely colored fruits and vegetables so that even if some of the pigment is destroyed, a sufficient portion may remain to impart the characteristic color of the product.
2. Discoloration during handling and transportation of the fresh produce should be avoided (Markakis & Jurd, 1974).
3. Inactivation or inhibition of enzymes involved in anthocyanin degradation is possible. Siegel et al.(1971) showed that blanching red tart cherries in live steam (100°C) for 45 to 60 sec prevented anthocyanin color loss in red tart cherries, which were subsequently stored frozen (-20°C) for 10 months and then thawed under conditions conducive to large pigment losses (up to two thirds for unblanched controls). It was also possible to inhibit the enzymatic degradation of anthocyanin in red tart cherry juice by adding 30 ppm of sulfur dioxide (Goodman and Markakis, 1965).
4. Avoiding or restricting contact with oxygen during processing and storage of the commodity will afford considerable protection of the anthocyanin pigment. Since oxygen is detrimental to other quality attributes of foods, packing under anoxic conditions may have multiple justification. The use of antioxidants, such as cynteine (Adams and Ongley, 1972) sulfur dioxide (alone or in combination with ascorbic acid),

rutin (Kyzlink et al., 1973), propyl gallate, and quercetin (Markakis et al., 1957) has also been reported as exerting a stabilizing effect on the anthocyanins of heat-preserved red fruits.

5. Lowering the pH within acceptable limits may contribute to the stabilization of anthocyanin pigments. Citric acid appears to be a particularly effective acidulant since it is also a metal chelating agent. (Markakis & Jurd, 1974)

6. The use of cans lined with special enamels is sine qua non in the canning of anthocyaninpigmented products. The can manufacturing companies will recommend the proper type of cans when they know the product to be packed. Interestingly, the addition of stannic chloride (0.2%) and, to a lesser extent, of stannous chloride or aluminum chloride was reported to stabilize the color of strawberry puree when a Color Difference Meter was used in colorimetry (Sistrunk and Cash, 1970). When the actual anthocyanin content of the puree was measured, however, it was found that the pigment concentration was "of the order of the control" (no salts added) (Wrolstad et al., 1973). It was suggested that the metal ions reacted with other strawberry constituents, possibly leucoanthocyanins, to form insoluble red chelates. The anthocyanin chiefly involved in the two aforementioned experiments contains no ortho-hydroxyl system. Cyanidin glycosides, on the other hand, do contain an orthohydroxyl system in the B ring and form relatively stable polyvalent metal complexes which have altered color characteristics (bluing).

7. Since heat is detrimental to anthocyanin pigments, the minimum heat treatment necessary for preservation of the product should be applied. In fact, Mackinney et al. (1955) proposed a process in which strawberry preserves are made at temperatures below 95°F (35°C), followed by pasteurization. On theoretical grounds a short-time-hightemperature treatment should result in better anthocyanin retention than a conventional heat treatment (Markakis et al., 1957). Quick cooling is recommended, as well as storage of the finished product at refrigeration temperatures, if possible. Adams and Ongley (1972) demonstrated that canned red fruits may retain 50% of the original anthocyanin.

2.2.4 Extraction and Analysis of Anthocyanins:

Extraction of the pigment from the raw material may be considered as the first major purification step (Markakis, 1982b)

Extraction is usually achieved by (a) water, (b) water containing SO_2, and (c) acidified alcohols. Hot water was used by Esselen and Sammy (1973, 1975) to extract the red cyanidin and delphinidin mono- and biosides of the calyces of roselle (*Hibiscus sabdariffa*). Palamidis and Markakis (1975) compared the extraction of grape wine pomace with hot water versus the extraction with a 500-ppm $S0_2$ solution. The SO_2 extract contained purer anthocyanin than the water extract; also, the anthocyanin of the SO_2 extract was more stable as a colorant of a soft beverage than the anthocyanin of the water extract. Extraction of anthocyanins is commonly conducted with methanol or ethanol containing a small amount of acid (15% 1.0 mol/L HCl) with the objective of obtaining the flavylium cation form, which is stable in a highly acid medium. Philip (1974) reported a process for recovering the anthocyanin present in grape wastes by extraction with methanol or ethanol containing 0.1 to 1.0% tartaric acid. The excess tartaric acid can be removed from the extract as insoluble potassium hydrogen tartarate by adding a calculated amount of 40% KOH. According to Francis (1982).With foods, 1% HC1 in ethanol may be preferred, due to the toxicity of methanol, even though it is slightly less effective in extraction and more difficult to concentrate.

Fractionation, separation and analysis of anthocyanins may be achieved through several techniques which include thin layer chromatography (TLC) (Strack and Wray 1989), several column chromatographies (CC) (Rivas-Gonzalo, 2003), high performance liquid chromatography (HPLC) (Wulf and Nagel, 1978; da Costa et al., 2000), high-speed counter current chromatography (HSCCC), high performance centrifugal partition chromatography (HPCPC) (Foucault and Chevolot 1998; Degenhardt and Winterhalter 2001; Schwarz et al., 2003), capillary electrophoresis (CE) and capillary zone electrophoresis (CZE) (Ichiyanagi et al., 2000). Different gels have been used in order to separate anthocyanins like silica gel, reversed-phase silica-gel, different kinds of polyamide, polymeric resins, etc.

The detection of anthocyanins can be achieved using UV-Vis spectroscopy (Wulf and Nagel, 1978), spectrofluorometry, mass spectrometry (FAB-MS, HPLC-MS, ESI-MS) (Lopes da Silva et al,. 2002; Mateus et al., 2002a; Favretto and Flamini, 2000), NMR (Fossen and Andersen, 1999) or infrared spectroscopy (Dambergs et al., 2006). The

coupling of different analytical techniques allowed improving the separation as well as the sensitivity of detection of anthocyanins. HPLC coupled to nuclear magnetic resonance (NMR) is one of the recent examples of such achievement (Wolfender et al. 1998; Wolfender et al. 2003; Stintzing et al., 2004).

2.2.5 Health and Physiological Effects:

Numerous studies on anthocyanins have shown that they are not only beneficial for food applications but also as therapeutic agents. As mentioned previously, anthocyanins exhibit a wide range of therapeutic effects which have been demonstrated in various in vitro systems, animal and human trials. These therapeutic properties will be discussed in the following sections.

2.2.5.1. Antioxidative Effects:

Dietary antioxidants can help combat reactive oxygen species and free radicals and help decrease the risk of chronic diseases such as coronary heart disease and certain cancers (Abdel et al., 2008). Anthocyanins have shown to be potent antioxidants, superior to well-known antioxidants such as butylated hydroxyanisole (BHA), alpha-tocopherol, 6-hydroxy-2,5,7,8-tetramethychromane-2-carboxylic acid (Trolox), catechin and quercetin (Kahkonen and Heinonen, 2003).

2.2.5.1.1 Radical Scavenging:

It has been observed that the anthocyanidins/anthocyanins with the highest antioxidant activity are delphinidin, cyanidin and cyanidin-3-glucoside. These are also significantly higher than the standards alpha-tocopherol, BHA, butylated hydroxytoluene (BHT) and ascorbic acid (Fukumoto and Mazza, 2000). The compounds with the lowest antioxidant activity are malvidin 3,5-diglucoside and pelargonidin 3,5-diglucoside. Monoglucosides generally have a higher scavenging capacity than the diglucosides. These results are similar to the findings obtained by Kahkonen and Heinonen (2003) and Astadi et al. (2009) who also found that anthocyanins possessed higher radical scavenging activity compared to the reference antioxidants (ascorbic acid, alpha-tocopherol, Trolox, BHT and rutin) in the DPPH test.

2.2.5.2 Anti-Obesity and Anti-Diabetic Properties:

2.2.5.2.1 Lipid Lowering and Oxidative Stress Properties:

Several studies have associated a diet high in fat with the development of type-2-diabetes and to induce hyperglycemia, hyperinsulinemia and hyperlipidemia. Recently, DeFuria et al., (2009) demonstrated that blueberries can protect against whole-body

insulin resistance by inhibiting the early inflammatory events in adipose tissue and can also improve glycemia in an animal model.Tsuda et al., (2003) found that anthocyanins (cyanidin-3-glucoside) from purple corn (2 g/kg diet) significantly suppressed the development of obesity and decreased hyperglycemia induced by a high fat diet in mice.

2.2.5.2.2. Insulin Secretion:

Jayaprakasam et al. (2005) investigated the glucose-induced insulin release from pancreatic beta-cells by anthocyanins and anthocyanidins in vitro, particularly the cyanidin, delphinidin and pelargonidn glycosides since they are the primary bioactive components of *Cornus* fruits (European and Asiatic Cornelian cherry). The results suggest that both anthocyanins and anthocyanidins are insulin secretagogues (enhance secretion).

2.2.5.3. Anticarcinogenic Effects:

Fimognari et al. (2004) identified cyanidin-3-glucoside as an anthocyanin compound capable of inducing apoptosis and cytodifferentiation in different leukemic cell lines. Additionally, treatment with cyanidin-3-glucoside was found to revert human melanoma cells from the proliferating to the differentiated state (Serafino et al., 2004). Anthocyanins have also been shown to prevent skin cancer in rodents. Topical application of anthocyanin-containing pomegranate extract elicited a delay in onset and decrease in incidence and burden of skin tumours (Harris et al., 2001).

2.2.6 Anthocyanins as a Food Colorant:

The use of unnatural additives is becoming less popular among the consumers, especially due to psychological reasons as the consumer easily associates natural colorants to health benefits and synthetic colorants to toxic issues. Therefore, replacing synthetic dyes by natural colorants has become a major issue over the last years. Pigments from natural sources may display a wide range of colors and are usually safe. Among those pigments widespread in nature, anthocyanins assume a crucial role when dealing with natural colorants. A good review of anthocyanins as food colorants is the one made by Francis (1989).

2.3 Pomegranate:

The pomegranate *(Punica granatum* L.) is one of the oldest fruits that has not changed much throughout the history (Damania, 2005; Heber, 2006). Numerous historical evidences suggest that this fruit was one of the first 5 crops along with Figure 2,

Dates, Olives and Grapes to be cultivated. Its domestication started 3000 -4000 BC in the North of Iran and Turkey (Lye, 2008) from where it spread to other regions e.g. Mediterranean countries, India and China, possibly through ancient trade routes. The pomegranate cultivation and usages are deeply embedded in human history and its utilization has been found in many ancient cultures as food as well as a medical remedy (Holland and Bar-Ya'akov, 2008) .In the Greek mythology it represents life, regeneration and marriage and in Persian mythology Isfandiyar (legendary Persian hero) eats a pomegranate and becomes invincible. In "The Persian Wars" Herodotus mentions "golden pomegranates" adorning the spears of warriors in the Persian phalanx; in Judaism pomegranate seeds are said to number 613-one for each of the Bible's 613 commandments; in Buddhism it is one of the blessed fruits and represents the essence of favourable influences; in China it is widely represented in ceramic art symbolising fertility, abundance, posterity, numerous and virtuous off springs and a blessed future; in Christianity it is a symbol of resurrection and eternal life; and in Islam the heavenly paradise of the Quran describes four gardens with shade, springs, and fruits including the pomegranate (Langley, 2000).

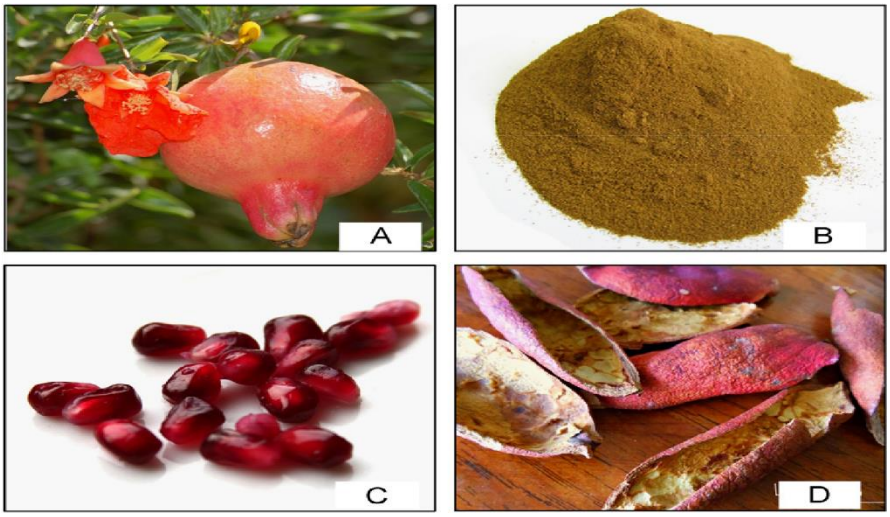

Figure 2: Pomegranate fruit (A) and its anatomical components, pomegranate peel powder (B) pomegranate seeds (C) and sundried pomegranate peel (D)

2.3.1 Traditional medicinal uses:

A variety of cultures and traditions in both the developing and developed worlds recommend pomegranate peel to treat common health problems. Traditionally, aqueous PoP extract is obtained by boiling for 10–40 min. The extract has been used to treat

diarrhea, dysentery, and dental plaque, in addition to being used as a douche and enema agent (Lansky et al., 2004). Similarly, diarrhea, intestinal worms, bleeding noses and ulcers have been treated in Indian Subcontinent with dried PoP, plant bark and flower infusions. PoPx is gargled as a liquid to relieve sore throat and hoarseness. Topical application of the rind powder can aid in healing bleeding gums and plaque in patients with periodontitis (Amrutesh, 2011). Oral ingestion of 5–10 g of peel powder is recommended two to three times a day for the treatment of hyperacidity.

2.3.2 Bioactivities of pomegranate peel ellagic acid and punicalagin:

The antioxidant activity of PoP is associated with its phenolic compounds in the form of anthocyanins, gallotannins, ellagitannins, gallagyl esters, hydroxybenzoic acids, hydroxycinnamic acids and dihydroflavonol, however, ellagitannins characterized by ellagic acid, gallic acid and punicalagin are the predominant phenolics of the fruit (Cerda et al., 2003; Larrosa et al., 2006). Ellagic acid occurs in free and bound forms (EA-glycosides and ellagitannins). The efficacy of ellagic acid as a tool to treat some low to mild and chronic disorders with very low rates of progression has been widely documented in the literature. Moreover, it has been shown to be a potential candidate as a chemopreventive agent for cancer treatment (Kelloff et al., 1994). Beside its other well-known ethnopharmacological properties, ellagic acid has been demonstrated to reduce white fat deposits and triglycerides levels accumulated in the body during regular intake of high-fat diets (Lei et al., 2007).

2.3.3 Peel phenolics extraction modeling:

Industrial scale extraction of phenolic compounds from PoP is carried out by using solvents such as methanol, ethanol, acetone, chloroform and ethyl acetate. Polar solvents have greater antioxidant extraction capability compared to non-polar solvents. The use of different solvents other than water for peel phenolic extraction are reported to yield different phenolic content ratios and associated antioxidant activity (Negi and Jayaprakasha, 2003; Negi et al., 2003; Zahin et al., 2010). Phenolics extracted from dried PoP with ethyl acetate, acetone, methanol and water demonstrate higher antioxidant activity, but aqueous extracts exhibit higher anti-mutagenic activity than methanolic extracts. Due to the adequate polarity of methanol, methanolic extracts of pomegranate peel possess higher antioxidant activity than other solvents (Ajaikumar et al., 2005; Iqbal et al., 2008; Negi et al., 2003; Singh et al., 2002; Zahin et al., 2010).

2.3.4 Nutraceutical properties of pomegranate and peel extracts:

2.3.4.1 Cardiovascular Protective role:

Atherosclerosis is one of the leading causes of death, particularly in developed countries where a higher percentage of atherosclerotic deaths are observed. Low density lipoproteins (LDL) accumulate in the interior layers of blood vessels and then undergo oxidation, a process that generates harmful species. Inhibition of LDL oxidation is considered to be a good strategy to prevent the accumulation of foam cells and, ultimately, cholesterol deposits in the arteries. Due to its excellent antioxidant activity, PoPx has the potential to inhibit LDL oxidation and thus retard the progression of atherosclerosis with a significant reduction of arterial foam cell levels. The pomegranate polyphenols punicalagin, gallic acid, and to lesser extent ellagic acid, increase hepatocyte paraoxonase 1 expression and secretion in a dose-dependent manner, thereby reducing the risk of atherosclerosis development (Khateeb et al., 2010; Rosenblat and Aviram, 2009).

2.3.4.2 Anti-inflammatory and anti-allergic properties:

The weight of compelling scientific evidence regarding the therapeutic benefits of pomegranate and its fractions has built a scientific consensus that pomegranate rind methanolic extract has the ability to inhibit inflammation and allergies (Panichayupakaranant et al. 2010b). Evidently, inflammatory cells including neutrophils, macrophages and monocytes may inflict damage to nearby tissues, an event thought to be of pathogenic significance in a large number of diseases such as emphysema, acute respiratory distress syndrome, atherosclerosis, reperfusion injury, malignancy and rheumatoid arthritis (Babior, 2000).

2.3.4.3 Anticancer properties:

ROS represent a causal and/or concausal factor in the development of cancer. Extensive ROS damage to DNA ultimately leads to somatic mutations and organ malignancies. At the cellular and tissue level, copper and iron binding sites of macromolecules serve as central sites for the production of free radicals. Such sitespecific free radical generation is inhibited by chelation of metal ions by antioxidants of biological origin, e.g., flavonoids (Chevion, 1988; Sies, 1997). Moreover, ellagic acid and punicalagin arrest cancer cell growth by inducing apoptosis-a multistep cell death program. Antioxidants, either from synthetic or plant sources, are thought to be a preventive

approach against cancerogenesis. Natural antioxidants, despite the presence of synthetic ones in the market, have gained a wide acceptance due to their high safe edible limits.

2.3.4.6 Antimicrobial potential of peel extracts:

In vivo and in situ application of an 80% methanolic extract of PoP presented an inhibitory effect against Listeria monocytogenes, Staphylococcus aureus, Escherichia coli and Yersinia enterocolitica (Al-Zoreky, 2009).

2.3.4.7 Wound healing potential:

Epithelialization, antioxidant immunity and characteristic biochemical properties are the common features of the wound healing process that prevail in injured tissues. Topical administration of PoPx can be recommended for dead tissue, incisional and excisional wound models. Improved epithelialization, breaking strength and contraction of incised wounds, along with increased hydroxyproline content, dry weight and breaking strength of granulated tissues, can be observed in the healing process of PoPx-treated wounds. In studies, oral administration of a 100 mg/kg aqueous extract of PoPx to Wistar rats and topical application of PoPx formulated with hydrophilic gel resulted in significant improvement in all wound models (Adiga et al., 2010; Murthy et al., 2004).

Chapter 3: Material and method

3.1 Sample collection:

Pomegranae (Punica granatum L), apple (*Malus pumila*) and commercial apple jelly were collected from the local market of Chittagong, Bangladesh. All of the experiments were held in the laboratory of Food Processing and Engineering and at the Dept. of Applied Food Science and Nutrition at Chittagong Veterinary and Animal Sciences University (CVASU).

3.2 Sample preparation:

The pomegranate was washed by tap water to remove adherences, dirt and other surface impurities. Then thin peels of pomegranate were taken manually with a stainless steel knife and cut into small pieces.

3.3 Chemicals and reagents:

1. Absolute ethanol,
2. DPPH,
3. Methanol etc.

3.4 Extract preparation:

Pomegranates peels were washed with water, chopped into small pieces, transferred into respective beakers added with absolute ethanol, and left to shake on a shaker for 72 h at room temperature. The solvent was then separated from the plant residue by straining. The filtrate was collected and stored at room temperature while the residue was re-extracted twice, each time with fresh solvent. Finally, all the filtrates were combined and evaporated under reduced pressure at 60 °C using a rotary evaporator to obtain the crude extracts. The crude extracts were weighed and stored at 4 °C until further analysis. In time stability of anthocyanins extracts under the influence of frozen condition (-10^0C) and natural light was studied at the 3 days intervals. Stability of anthocyanins extracts under the influence of temperature was studied at 30^0C, 45^0C, 60^0C and 75^0C for 30 minutes in water bath.

In order to study the influence of natural light on stability of anthocyanins extracts some samples were stored in the light and in the dark.

3.5 Total anthocyanin content (TAC) assay:

TAC of the vegetable extracts was determined colorimetrically following the method described with slight modifications (Selim et al., 2008). Stock solutions of 10 mg/mL of extracts were prepared. Extract solution (3 mL) was pippetted into a cuvette. The

intensity of the extract color was measured at wavelength 520 nm using UV-VIS spectrophotometer (U-2600, Shimadzu). Ethanol was used as a blank. TAC was calculated and expressed as milligrams per 100 g (mg/100 g) using the following equation:

$$\mathrm{TAC} = \frac{\text{Absorbance of sample} \times \mathrm{DF} \times 100}{\mathrm{m} \times \mathrm{E}}$$

Where,

DF stands for dilution factor;

m means weight of sample used to make stock solution;

E refers to extinction coefficient (55.9).

Each experiment was replicated for three times.

3.6 Determination of antioxidant capacity:

Antioxidant capacity of the extracts was determined using DPPH assay as described by Azlim et al., (2010) with slight modifications. Stock solution (1 mg/mL) of extract was diluted to concentrations of 0.10, 0.20, 0.30, 0.40, 0.60, and 0.80 mg/mL in methanol. Methanolic DPPH solution was prepared by dissolving 6 mg of DPPH in 100 mL methanol. The methanolic DPPH solution (2 mL) was added to 1 mL of each extract solution of different concentrations and the mixture was left for 30 min and the absorbance will be read at wavelength 517 nm. Control was prepared by mixing 1 mL of methanol with 2 mL of DPPH solution. Methanol was used as a blank. Antioxidant capacity based on the DPPH free radical scavenging ability of extracts was calculated using the following equation:

% inhibition= (1-(Absorbance of sample/Absorbance of control)) ×100%

Each experiment was replicated for three times.

3.7 Analysis of minerals:

The contents of P, Fe, K, Ca and Mg were measured by Biochemical Analyzer, (Humalyzer 3000) commercially available biochemical kit (Randox®) was used for biochemical assay.

3.7.1 Sample preparation:

0.10 g of each sample (natural and artificial color) was taken to dissolve in 10 ml of distilled water. Then it was filtered through muslin cloth and further filtered by whatman paper. Finally it was used for mineral analysis.

3.7.2 Potassium (K):

3.6.2.1 Test Principle:

Sodium tetraphenylboron reacts with potassium in the sample to produce a fine turbidity of potassium tetraphenylboron. The intensity of turbidity is directly proportional to the concentration of potassium in the sample.

3.7.2.2 Reagent composition:

A. Potassium TPB reagent: Sodium tetraphenylboron 0.2 mol/L, sodium hydroxide 2.2 mol/L, preservative 0.1`%

B. Potassium standard: Potassium 5 mmol/L

3.7.2.3 Assay:

Wavelength / filter: 630 nm (Hg 623) /Green

Temperature : Room Temperature

Light path : 1 cm.

3.7.2.4 Pipetting Scheme:

Pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	**B (ml)**	**Standard(ml)**	**Sample (ml)**
Potassium Reagent	1.0	1.0	1.0
Deionized Water	0.02	-	-
Potassium Standard(S)	-	0.02	-
Sample	-	-	0.02

Mixed well and incubated at R.T. for 2 minutes. The absorbance of the standard (Abs.S) and Test Sample (Abs.T) was measured against Blank, within 60 minutes.

3.7.2.5 Calculation:

$$\text{Potassium(mg/dl)} = \frac{\text{(B) Sample}}{\text{(B) Standard}} \times \text{Standard conc.(mg/dl)}$$

3.7.3 Calcium (Ca):

3.7.3.1 Reaction principle:

Calcium ion forms a violet complex with O-Cresolphthalein complexion in an alkaline medium.

3.7.3.2 Reagent composition:

A. Standard calcium

B. Buffer:2-amino-2-methyl –propan –lol 3.5 mol/l

C. Chromogen: O-Cresolphthalein complexone 0.16 mmol/l,8-Hydroxyquinoline 6.89 mmol/l, Hydrochloric acid 60 mmol/l

D. EDTA 150mmol/l

3.7.3.3 Assay:

Wavelength / filter: Hg 578 nm (550-590)

Pectrophotometer: 570nm

Temperature : 20-25^{O}C / 37^{O}C

Light path : 1 cm.

3.7.3.4 Pipetting Scheme:

Pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	Reagent Blank	Standard	Sample
Working Reagent (ml)	1.0	1.0	1.0
Deionized Water (µl)	25 µl	-	-
Standard (S) (µl)	-	25 µl	-
Sample (µl)	-	-	25 µl

Mixed well and incubated at R.T. for 2 minutes. The absorbance of the standard (Abs.S) and Test Sample (Abs.T) was measured against Blank, within 60 minutes.

3.6.3.5 Calculation:

$$\text{Calcium (mg/dl)} = \frac{\text{(C) Sample}}{\text{(C) Standard}} \times \text{Standard conc. (mg/dl)}$$

3.7.4 Magnesium (Mg):

3.7.4.1 Principles:

The method is based on the specific binding of calmagite,a metallochrome indicator and magnesium at alkaline pH with the resulting shift in the absorption wavelength of the complex. The intensity of the chromophore formed is proportional to the concentration of magnesium in the sample.

Calgamite+Magnesium ⟶ Calmagite magnesium complex

3.7.4.2 Assay:

Wavelength:	520 nm, Hg 546 nm 500-550 nm (Increase of absorbance)
	628 nm, Hg 623 nm, 570-650 nm (Decrease of absorbance)
Cuvette:	1 cm light path
Temperature:	20-25^{O}C / 37^{O}C
Measurement:	Against reagent blank

3.7.4.3 Pipetting Scheme:

Addition sequence	**Blank**	**Sample or Standard**
Sample or Standard	-	10 µ
Dist. Water	10 µl	-
Reagent	1000 µl	1000 µl

Mixed and absorbance was taken against blank after 5-60 min. at 20-25^{O}C / 37^{O}C.

3.7.4.4 Calculation:

$$\text{Magnesium (mg/dl)} = \frac{\text{(D) Sample}}{\text{(D) Standard}} \times \text{Standard conc. (mg/dl)}$$

3.7.5 Determination of iron (Fe):

3.7.5.1 Reaction principle:

The iron is dissociated from transferring iron complex in weakly acid medium. Liberated iron is reduced into the bivalent form by means of ascorbic acid. Ferrous ions give with Ferro Zine a colored complex.

Transferrin (Fe^{3+}) 2+e $\longrightarrow$ $2Fe^{3+}$+Transferrin

Fe^{2+} $\longrightarrow$ Colored complex

3.7.5.2 Reagent composition:

Reagent	**Component**	**Concentration**
Buffer	Acetate pH 4.9	100 mmol/L
Reductant	Ascorbic acid	99.7%
Color	Ferrozine	40 mmol/L
Iron cal.	Iron aqueous primary standard	100 µg/dl

3.7.5.3 Assay:

Wavelength	**562nm (530-590)**
Cuvette	**1 cm light path**
Temperature	**37ºc/15-25ºc**

3.7.5.4 Pipetting Scheme:

Pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	**Reagent Blank**	**Standard**	**Sample Blank**	**Sample**
WR (ml)	**1.0**	**1.0**	**1.0**	**1.0**
Ferrozine	**1**	**1**	**-**	**1**
Distilled water(µl)	**200**	**-**	**-**	**-**
Standard (µl)	**-**	**200**	**-**	**-**
Sample (µl)	**-**	**-**	**200**	**200**

Mixed well and incubated at R.T. for 2 minutes. The absorbance of the standard (Abs.S) and Test Sample (Abs.T) was measured against Blank. The color is stable for at least 30 minutes.

3.7.5.5 Calculation:

$$\text{Iron } (\mu g/dl) = \frac{(E)\text{Sample} - (E)\text{Sample Blank}}{(E)\text{Standard}} \times 100 \text{ (Standard conc.)} \mu g/dl$$

Conversion factor: µg /dl×0.179=µmol/l

3.7.6 Determination of phosphorus:

3.7.6.1 Principle:

Phosphorus ions in acidic medium react with ammonium molybdate to form a phophomolybdate complex.This complex has an absorbancein the ultraviolate range and is measured at 340nm. Intensity of the complex formed is directly proportional to the amount of inorganic phosphorus present in the sample.

Phosphorus+Ammonium Molybdate ⟶ Phosphomolybdate

3.7.6.2 Assay:

Wavelength:	**340 nm, (Hg 365 nm)**
Cuvette:	**1 cm light path**
Temperature:	**Room temperature**

3.7.6.3 Reagent composition:

Acid reagent

Molybdate Reagent

Phosphorus standard (5mg/dl)

3.7.6.4 Pipetting Scheme:

Addition Sequence	Reagent Blank	Standard	Sample
Working Reagent (ml)	1.0	1.0	1.0
Distilled Water (ml)	0.01	-	-
Standard (S) (ml)	-	0.01	-
Sample (ml)	-	-	0.01

Mixed well and incubated at R.T. for 5 minutes. The absorbance of the standard (Abs.S) and Test Sample (Abs.T) was measured against Blank, within 60 minutes.

3.7.6.5 Calculation:

$$\text{Phosphorus (mg/dl)} = \frac{\text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Standard}}} \times \text{Standard conc (mg/dl)}$$

3.8 Preparation of apple jelly with natural and artificial color (mixture of E110, E122 and E330):

Apple jelly was prepared by using method by (Panchal et al., 2018).The clear fruit (250 ml) extract was poured in a stainless steel pan and boiled, then required amount of pectin (4.9g) was mixed with small amount sugar (40g) in a stainless steel pot. The remaining sugar (100g) was mixed with fruit extract and mixture was boiled until the TSS become nearer to 55 °Brix. Then sugar mixed pectin was added and continued the boiling until TSS becomes nearer to 58 °Brix. The citric acid was added (1.25g) and continued the boiling till the desired consistency and TSS reaches to 67° Brix. Then the sodium benzoate and small amount of natural color were added respectively. Jelly for artificial color was made in the same way.

3.9 Sensory evaluation of jelly:

The Sensory evaluation of apple jelly samples (sample with natural color, sample with artificial color and commercial jelly) were carried out according to the standard method of Amerine et al. (1965) on 9 point Hedonic scale, The mean score minimum 10 semi trained judges for each quality parameter viz., color and appearance, taste, flavor, consistency, transparency and overall acceptability was recorded.

The scale were arranged such that: Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, Neither like nor dislike = 5, Dislike slight = 4, Dislike moderately = 3, Dislike very much = 2, Dislike Extremely = 1. This method does not, of course, reflect actual consumer perception, but it does strongly indicate attributes which a good quality product should possess (Sing et al., 2008).

3.10 Statistical Analysis:

Data collected in this study was analyzed by one way (Tukey's Multiple Comparison Test) using SAS (Statiscally analysis system) 9.3 and SPSS (Statistical Package for the Social Sciences) version 16.0. A significant difference was considered at the level of $p<0.05$.

Chapter 4: Result

4.1 Total anthocyanin content (TAC) in mg/100g at frozen storage and influence of light

One way ANOVA (Analysis of variance) test (Table 3) was performed to see the overall mean difference of days for frozen storage, presence of light and absence of light.The result showed that there was highly significant mean difference of days on anthocyanin in different situation (frozen storage, presence of light and absence of light).Tukey's multiple comparison test was performed to make sure for which days were the most significant for anthocyanin.It was observed that all pairwise comparisons were significantly different from one another regarding to frozen. Regarding to presence of light, all pairwise comparison were significant. Regarding to Absence of light, Tukey's Studentized range test was performed and the result observed that all pairwise days combination were significant except the days of 3 and 6.Two sample independent mean (t test) was done to evaluate any mean significant difference between presence and absence of light for anthocyanin content, it was observed that there was no significant mean difference between presence and absence of light of anthocyanin content (t test value 0.88 and P-value=0.40).

Table 3: Total anthocyanin content (mg/100g) at frozen storage (at -10^{0}C) and the influence of light

Total Anthocyanin Content (mg/100g) at -10^{0}C			
Days	Frozen storage (Mean±SD)	Presence of light (mean±SD)	Absence of light(mean±SD)
0	48.3±0.4^a	48.3±0.4^a	48.3±0.4^a
3	32.22±2.02^b	39.61±1.71^b	41.7±2.98161^b
6	25.06±1.16^c	32.48±0.78^c	35.8±2.57099^b
9	17.89±1.29^d	26.63±1.60^d	32.22±1.38^c
F test	280.20**	167.76**	33.93**

** Significant at P <0.01; Values followed by different superscript letters denote a significant difference; comparison done across the days.

Results are means ± standard deviation of triplicates (n=3)

4.2 Total anthocyanin content (TAC) in mg/100g at at different heating temperature

One way ANOVA (Analysis of variance) test was performed to see the overall mean difference of temperature for degradation of anthocyanin (Table 4). It was found from the table that there was highly significant mean difference of temparature on anthocyanin degradation. Tukey's multiple comparison test was performed to make sure for which temparatures were the most significant for anthocyanin. It was observed that control (no exposure) was significantly different from 30^0, 45^0, 60^0 and 75^0C temperature, 30^0 and 75^0 were most significant for degradation of anthocyanin. There was no significant difference between 30^0 and 45^0 C; 30^0 and 60^0 C; 45^0 and 60^0C; and vice-versa.

Table 4: Total anthocyanin content (TAC) in mg/100g at different heating temperature

Heating temp (^{0}C)	Total anthocyanin content(mean±SD)
control	48.3 ± 0.4^{a}
30	34.01 ± 3.53467^{b}
45	30.43 ± 3.61316^{bc}
60	26.85 ± 2.55^{bc}
75	23.37 ± 3.17^{c}
F test	32.960**

** Significant at P <0.01; Values followed by different superscript letters denote a significant difference; comparison done across temperatures.

Results are means ± standard deviation of triplicates (n=3)

4.3 Antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay:

Figure 3 shows the % inhibition of the natural color extracted from pomegranate peel against the concentration (mg/ml). From the figure it can be showed that the increase in the % inhibition with the dose.

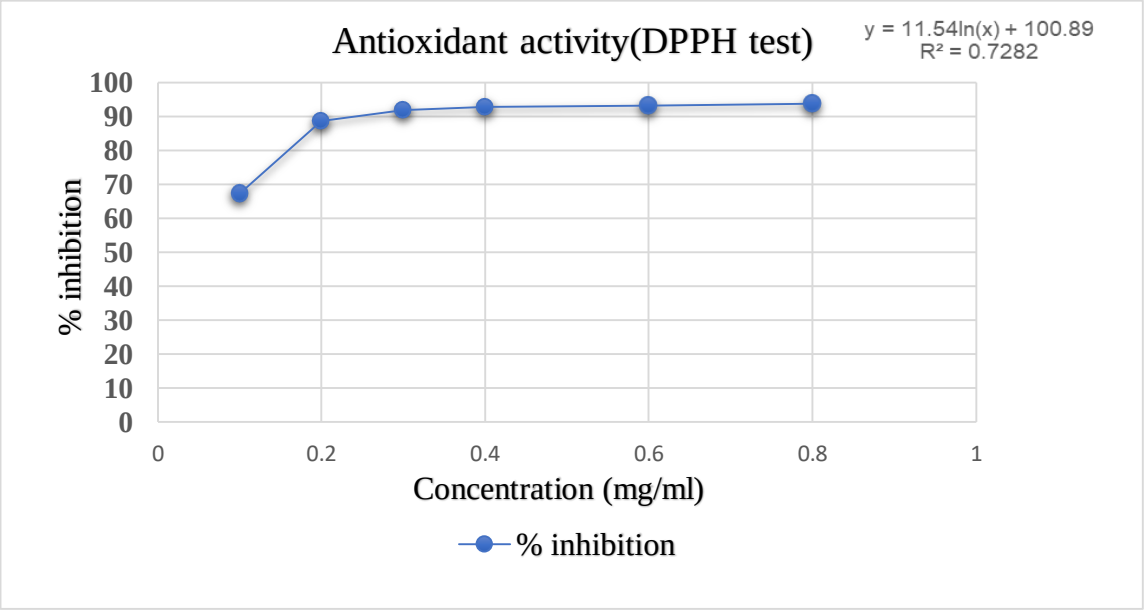

Figure 3: The dose-response curve for antioxidant activity of pomegranate peel color against concentration (mg/ml) by DPPH method

The % inhibition was sharply increased (67.31%) initially (0.1 mg/ml) and increased slowly latter (Figure 3). From the Figure it is shown the % inhibition at concentration 0.1 mg/ml is significantly different from and 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml and vice versa. The % inhibition at 0.2 mg/ml is significantly different from 0.4 mg/ml, 0.6 mg/ml and 0.6 mg/ml and vice versa (Appendix B).

The IC_{50} value of the natural color was 0.04 µg/ml calculated from this non linear regression curve.

4.4 Analysis of Minerals:

Mineral content (Mg, P, K, Fe, Ca,) for natural and artificial color was tested by two independent sample mean‘t’ test and the test value is 6.15 and P-value is 0.03, which mean that the mean mineral content of natural and artificial color was significantly different (Figure 4).

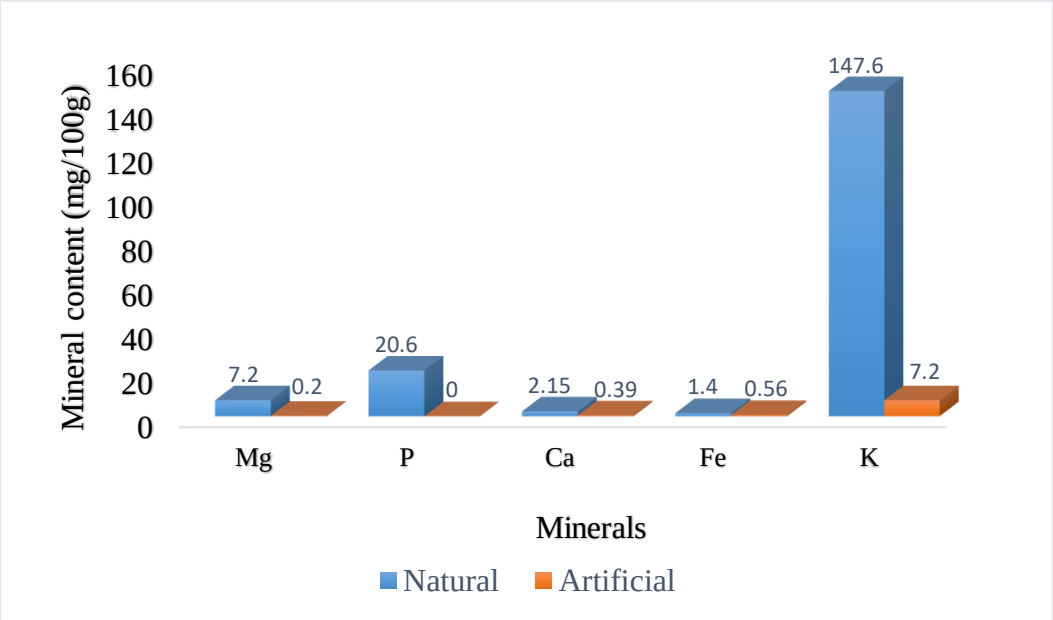

Figure 4: Mineral content (mg/100 g) of Natural and artificial colors

4.5 Sensory Analysis of jelly with natural color extracted from pomegranate peel, artificial color and commercial jelly:

The mean score for appearance, color, flavor, texture and overall acceptability of the jellies were evaluated and the mean score of their responses are represented in Table 5

It was observed that the mean scores of hedonic scales were significantly different for flavor, color, texture and taste separately for samples. The mean score of color, appearance and general acceptance were not significantly different and the multiple Tukey's Multiple Comparison Test (TMCT) at ($p<0.05$) was performed to show the pairwise significant difference of flavor, texture and taste of different categories. It was observed that regarding to flavor of jelly with natural (SN) and commercial food color (SA) was not different. Regarding to texture, jelly with natural (SN) and artificial color (SA) were significantly different and jelly with artificial color (SA) and commercial jelly (SC) were significantly different. Regarding to taste for different category of jelly, the jelly with natural color (SN) and commercial jelly (SC) were significantly different and jelly with artificial color (SA) and commercial jelly (SC) were significantly different.

The result (Table 5) indicates that the color of the sample with natural color (SN) was in highest position (8.2).

The significant flavor differences ($p<0.05$) were revealed among the samples of jelly (Table 5) were equally acceptable and significantly differs from others. The flavor score of sample with natural color (SN) was the highest score (8.07) among the samples of the jellies while the commercial sample (SC) obtained the lowest score (6.6) among the samples.

The data shows that, sample with natural color (SN) possesses highest score (8.1) in case of texture and the lowest score (7.0) was obtained the sample with artificial color (SA).

In case of taste preference among the samples, a one way ANOVA showed that the samples were highly significantly different ($p<0.05$). The data shows that, sample with natural color (SN) possesses highest score (8.5) in case of taste and the lowest score (6.6) was obtained the commercial sample.

The reliability test was done by intraclass correlation coefficient. It was observed that the intra-rater agreement was higher for appearance (0.630) and general acceptance (0.541) and significant. On the other hand, intra-rater agreement was lower for color, flavor, texture and taste and not significant.

Table 5: Sensory Analysis of jelly

Sl. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean Score±SD	F test	Intra-rater reliability
Color	SN	9	9	9	8	8	7	9	9	8	8	7	8	8	8	8	8.2 ± 0.68	0.757^{NS}	0.223 (0.275)NS
	SA	8	8	9	9	8	7	8	8	7	7	8	7	6	9	8	7.8 ±0.86		
	SC	9	7	9	4	7	8	8	9	9	9	8	3	8	9	8	7.7 ±1.84		
Flavor	SN	9	9	8	9	8	7	8	8	8	8	8	9	6	8	8	8.07 ± 0.8^{a}	4.22*	0.167 (0.631)NS
	SA	8	9	9	8	8	8	8	7	7	6	7	6	6	8	8	7.7 ± 0.9^{ab}		
	SC	4	6	6	4	7	8	9	8	9	7	6	1	7	9	7	6.6 ± 2.2^{b}		
Texture	SN	8	8	9	8	8	8	9	8	9	7	8	8	8	8	8	8.1 ± 0.52^{a}	10.07**	0.324 (0.794)NS
	SA	7	9	9	9	8	7	8	7	7	6	8	8	8	7	7	7^{b}		
	SC	9	6	7	8	7	7	9	7	9	9	6	7	8	9	8	7.7 ± 1.1^{a}		
Taste	SN	9	9	9	9	9	8	9	8	9	7	8	9	8	8	8	8.5 ± 0.64^{a}	15.20**	0.172 (0.323)NS
	SA	8	9	9	9	9	8	8	8	7	8	7	8	7	9	8	8.1 ± 0.74^{a}		
	SC	4	8	6	6	7	8	8	6	8	7	5	7	8	7	4	6.6 ± 1.4^{b}		
Appearance	SN	8	9	7	9	8	7	8	8	8	8	6	8	8	8	8	7.9±0.74	2.47^{NS}	0.630 (0.012)*
	SA	8	7	7	9	8	8	9	8	8	7	6	6	7	8	7	7.5±0.92		
	SC	9	9	9	9	8	8	8	9	9	8	8	5	8	9	8	8.3±1.03		
General Acceptance	SN	9	9	8	8	8	7	9	8	8	8	8	8	8	8	8	8.1±0.52	2.67^{NS}	0.541 (0.039)*
	SA	8	9	9	9	8	7	8	8	7	7	6	6	7	8	7	7.6±0.99		
	SC	5	9	7	9	7	8	9	8	8	8	6	4	8	7	5	7.2±1.57		

NS=Not significant, * Significant at P<0.05, **Significant at P<0.01

Here,

SN= Sample of jelly with natural color, SA= Sample of jelly with artificial color and SC= Commercial sample of jelly

Chapter 5: Discussion

5.1 Effect of frozen storage and light on total anthocyanin content of color from pomegranate peel:

Anthocyanins, an antioxidant in peel, are the most important and abundant natural pigments belonging flavonoid family (Li et al., 2006). Evaluation of the frozen pomegranate peel color showed that total anthocyanins decreased by about 62.96% after 9th days' frozen storage (−10 °C) (Table 3).Similar effect was found in frozen pomegranate juice (mirsaeedghazi et al., 2011) in which total anthocyanins decreased by about 11% after 20 days' frozen storage (−25 °C) .These results showed that frozen storage, cannot preserve anthocyanin content of color extracted from pomegranate peel.

Frozen storage affects the stability of total anthocyanin content of fruits in different ways. Several studies show a decreasing trend in total fruit anthocyanins during frozen storage, e.g., in unclarified pomegranate juice (Turfani et al., 2012); strawberries (Holzwarth et al., 2012, Poiana et al., 2010a; Oszmianski et al., 2009; Ngo et al., 2007; Garcia-Viguera et al., 1999; Larsen and Poll, 1995; Deng and Ueda, 1993); black carrot juice (Turkyimaz and Ozkan, 2012); dates (Allaith et al., 2012); blackberries (Jacques et al., 2010; Poiana et al., 2010b; Kopjar et al., 2009); sweet cherries (Poiana et al., 2010a); blueberries (Poiana et al., 2010b; Srivastava et al., 2007); red raspberries (Poiana et al., 2010b; De Ancos et al., 2000); Myrtle berries (Tuberosco et al., 2008); blackcurrants (Hollands et al., 2008); and sour cherries (Urbanyi and Horti, 1992). A few studies found an increase in total anthocyanins during frozen storage, including red raspberries (Syamaladevi et al., 2011; De Ancos et al., 2000); serviceberries (Michalczyk and Macura, 2010); strawberries (Oszmianski et al., 2009); sour cherries (Urbanyi and Horti, 1992). This has been attributed to a concentration effect from moisture loss or enhanced extraction of anthocyanins due to tissue softening (Hager et al., 2008b). Some studies show no significant change in total anthocyanins during frozen storage of fruits, e.g., clarified pomegranate juice (Turfani et al., 2012), black carrot juice (Turkyimaz and Ozkan, 2012), blackberries (Hager et al., 2008a), black raspberries (Hager et al., 2008b), red raspberries (Sousa et al., 2005; Mullen et al., 2002), and blueberries (Lohachoompol et al., 2004).

It is not feasible to conduct a complete comparison between studies, since the fruit variety, maturity level, initial pH, storage temperature, and time differ. De Ancos et al. (2000) observed that frozen storage affects total anthocyanin content and individual

anthocyanin distribution in different ways, depending on the cultivar. Total anthocyanins in Heritage and Autumn Bliss raspberries were found to increase slightly, at 17% and 5%, respectively, after frozen storage for 360 days. The relative percentage of individual anthocyanins in the early raspberry cultivars, Heritage and Autumn Bliss, showed small changes during frozen storage, apparently due to increased extraction of the main pigments, cyanidin 3-sophoroside and cyanidin 3-rutinoside, during frozen storage. The total value of anthocyanins in late cultivars was significantly decreased (4–17%) in both Rubi and Zeva cultivars during frozen storage. These results could be explained by the different chemical compositions found between early cultivars (Heritage and Autumn Bliss) and late cultivars (Rubi and Zeva). Raspberry cultivars with low pH, high soluble solids content (°Brix), and high total anthocyanin content retained the initial anthocyanin concentration better during processing. Many studies have shown that long-term frozen storage has a significant impact on anthocyanins depending on the type of fruit. However, during the initial period of frozen storage (4 to 6 months), there is little or no significant decrease in total anthocyanins, with the rate of degradation increasing after this period (Poiana et al., 2010a,b; Chaovanalikit and Wrolstad, 2004; Sahari et al., 2004).

The effects of light on preparations containing anthocyanins is usually deleterious. From Table 3 it can be seen that the total anthocyanin content is decreased by 44.87% in the presence of light opposed to 33.29% in the absence of light after 9^{th} day of storage. Similar effect found by Laleh et al.(2006) for four Berberies species in which the destruction of total anthocyanin content were 85.22%, 79.04%, 59.22% and 26.4% respectively in the presence of light opposed to72.06%, 21.23%, 96.61% and 75.24% respectively in the absence of light for *B. integerrima, B. vulgaris, B. khorasanica* and *B. orthobotrys.* Stanciu et al., (2010) also found the influence of light exposure during one month on anthocyanins extracts (mg/100g fresh product) from black grape skin (*Vitis Vinifera*) in which degradation of anthocyanins content were 37.91% (861.206 to 534.947 mg /100g fresh product), 68.14% (500.267 to 159.376 mg/100g fresh product) and 89.48% (501.358 to 52.71 mg/100g fresh product) for three types of extracts respectively.

Tressler et.al. (1936) reported the adverse effect of light on the color of Concord grape juice in bottles. Van Buren and co-workers (1968) reported that the acylated diglucosides in wine exposed to light were the most stable. The monoglucosides were

the least stable and the nonacylated diglucosides were intermediate. Palamidis and Markakis (1975) reported that colorants extracted from grape pomace and formulated into a carbonated beverage showed increased degradation when exposed to light. At 20°C in the dark, the half-life was 416 d as opposed to 197 in daylight.

5.2 Effect of heating temperature on total anthocyanin content of color from pomegranate peel:

The rate of anthocyanin degradation increases with rising temperatures, as is the case with most chemical reactions (Markakis & Jurd, 1974).

Table 4 showed the total anthocyanin content (mg/100g) with the increase in heating temperature (30^0,45^0, 60^0 and 75^0C) which were 34.01 (mg/100g), 30.43 (mg/100g), 26.85 (mg/100g) and 23.37 (mg/100g).

Pure pelargonidin-3-glucoside, heated (50° to 110°C) in aqueous solution, loses color according to first-order reaction kinetics and with an activation energy of 27,000 cal/mol (Markakis, 1955). The same pigment in strawberry juice and preserves exhibits similar kinetic behavior (Meschter, 1953; Decareau et al., 1956). Meschter (1953) was able to demonstrate a logarithmic relationship between anthocyanin color retention in strawberry preserves and temperature, over a range covering both processing and storage temperatures. He showed that the time for 50% destruction (half-life) of anthocyanin pigment in a strawberry preserve at 100°C was 1 hr; during storage at 38°C (100°F, a common U.S. Army food storage temperature specification) the half-life was 10 days, at 20°C (68°F) it was 54 days, and at 0°C (32°F) the pigment half-life would be expected to be 11 months. Markakis et al., (1957) found that the temperature effect on pure pelargonidin-3-glucoside, the major strawberry pigment, in buffer solution was not very different from that described by Meshter and they recommended short-time-high-temperature heat processing for better pigment retention. These results were recently corroborated by Adams (1972 and 1973) and Ongley, who showed that during high temperature sterilization of canned and bottled red fruits the loss of pigment was negligible in comparison to that occurring during slow cooling and subsequent ambient temperature storage.

Maccarone et al., (1985) have studied the stability of anthocyanin in red orange juice in 15^0 C, 25^0 C and 35^0 C during a 15 day period and found that the increase intemperature accelerates the destruction of anthocyanins.

5.3 Antioxidant activity of natural color extracted from pomegranate peel:

One parameter that has been introduced recently for the interpretation of the results from the DPPH method, is the "efficient concentration" or EC_{50} value (otherwise called the IC_{50} value). This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color). (Molyneux et al., 2004)

Figure 3 shows the dose–response curve for the DPPH-scavenging effect of the pomegranate peel color. In addition, the efficient concentration of the test samples required to exhibit 50% of DPPH scavenging effect (IC_{50}) is 0.04µg/ml calculated by non linear regression curve.

Kanatt et al., (2010) found the IC_{50} value of Peel extract for DPPH radical scavenging was 4.9 µg mL^{-1} indicating that it was a stronger antioxidant.

Kasliwali and Quadri (2016) found IC_{50} Value 20mg/ml by DPPH assay. The results obtained from both research are higher than the current research. The reasons for these variations may be the cultivars, methodology, climatic condition etc.

5.4 Mineral analysis (Mg, P, Ca, Fe and K) of natural color extracted from pomegranate peel and artificial color (mixture of E110 Carmoisine, E122 sunset yellow and citric acid E330):

Figure 4 shows the significant difference of mineral content (mg/100g) between two colors (natural and artificial) at $p<0.05$ which shows 7.2 mg/100g Magnesium (Mg), 20.6 mg/100g Phosphorus (P), 2.15 mg/100g Calcium (Ca), 1.4 mg/100g Iron (Fe) and 147.6 mg/100g Potassium (K) for natural color whereas the content of these minerals were 0.2 mg/100g (Mg), 0.39mg/100g (Ca), 0.56 mg/100g (Fe) and 7.2 mg/100g (K) for artificial color. No Phosphorus (P) found in this artificial color. This variation might be due to their compositions.

Sharma et al., (2018) found 6679.5 mg/kg or 667.95 mg/100g Ca, 728.23 mg/1kg or 72.82 mg/100g Mg, 524.80 mg/kg or 52.48 mg/100g K, 57.01 mg/kg or 5.7 mg/100g phosphorus and 18.33µg/kg or 1.8mg/100g(closed to the result) for pomegranate peel extract powder. This variation between two results might be due to the solvent purity, extraction method, analytical methods, cultivars etc.

Al-Maiman and Ahmad (2002) reported high contents of Cu, Fe, Zn, Mg, P, Na, Ca and K in the seed and juice of "Taifi" cultivar cultivated in Saudi Arabia, while Mirdehghan

and Rahemi (2007) emphasised the high amount of some mineral nutrients in both peel and arils, especially microelements in the arils of Iranian cultivar “Malas Yazdi“.

Fawole and Opara, (2012) found overall, major mineral nutrient concentrations in the pomegranate rind or peel of some cultivars ranged from 146 (Arakta) to 351.50mg/kg (Bhagwa) for N, 12.31 (Ganesh) to 33.03mg/kg (Bhagwa) for P, 323.5 (Ganesh) to 554.0mg/kg (Bhagwa) for K, 16.15 (Arakta) to 60.65mg/kg (Ruby) for Ca, 10.50 (Ganesh) to 26.4mg/kg (Arakta) for Mg, 9.10 (Ganesh) to 19.43mg/kg (Bhagwa) for S, 39.78 (Ruby) to 122.96mg/kg (Bhagwa) for Cl, and 40.40 (Ganesh) to 117.40mg/kg (Wonderful) for Na. Mineral contents were determined in lower quantities in the mesocarp ranging between 108.5 (Arakta)205.5mg/kg (Herskawitz) for N, 13.83 (Ganesh) to 23.33mg/kg (Herskawitz) for P, 254.0 (Molla de Elche) to 394.5mg/kg (Herskawitz) for K, 7.5 (Molla de Elche) to 23.65mg/kg (Arakta) for Ca, 5.10 (Molla de Elche) to 10.35mg/kg (Arakta) for Mg, 6.91 (Ganesh) to 10.79mg/kg (Bhagwa) for S, 22.98 (Molla de Elche) to 57.68mg/kg (Bhagwa) for Cl, while Na content ranged between 24.75 (Ganesh) and 40.15mg/kg (Wonderful).

5.5 Sensory evaluation of jelly:

Sensory characteristics of the developed products and the commercial jelly showed that the overall acceptability of the samples got the hedonic scale like very much. The results indicate that the formulated jellies are equally acceptable since it got the same hedonic scale of that commercial sample SC, although no flavor were added to the formulated product which effects the score of the formulated product in terms of color and flavor (Table 5) . However, no significant difference in terms of appearance of the formulated products with the commercial products, which indicates positive sign for the developed product.

Chapter 6: Conclusion

Employing natural colors is the current marketing trend because of consumers concern about the safety of artificial food dyes, reinforced by possible health benefits of the natural pigments. Consumers are avoiding foods containing synthetic colorants, which lead food industries to replace them by natural pigments. Hence, it is worthwhile to review the research done so far in the field of natural colors. The pomegranate peels make up about 60% of the fruit, and they are rich in anthocyanin. The peel is the part of the fruit with the highest antioxidant activity.This is a beneficial fact of pomegranate peel to be used as a source for color rich in both anthocyanin and antioxidant. In such way, it can be added an economic value. Thus this research work would help in creating a new field of natural color for food sector with reducing unemployment problem in Bangladesh.

Chapter 7: Recommendations and Future perspectives

This study (The study on stability of anthocyanin of natural color extracted from Pomegranate peel) suggests following recommendations:

1. Different novel extracton technique such as ultrasonication assisted extraction techniques, microwave assisted Extraction can be used to extract color.
2. TAC (Total anthocyanin content) calculated by above method should be compared with other such as pH differential method to assess the difference between two methods.
3. Evaluation of other parameter of anthocyanin in different technologies in different condition such as pH, enzyme, oxygen, metal, solvent etc should be considered.
4. The toxicological test for the extracted color must be performed.
5. The color quality should be measured by different colorimetric method.
6. The result should be carried out for other natural sources in order to compare the results.
7. The analysis of stabilizing technique of the colorant should be performed.
8. The analysis of color of various cultivars of different food source can be performed to compare the results.
9. This study should be carried out for other colorants such as β-carotene, xanthophil, lycopene etc.
10. The most important thing is that this study should be carried out in a room with controlled atmosphere in order to obtain more specific results.

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Appendix A:

Total anthocyanin content (TAC) at Frozen Storage and the influence of light

Total anthocyanin content (TAC) at Frozen Storage

Days	Total anthocyanin content (mg/100g)
0	48.3 48.7 47.9
3	32.22 30.2 34.24
6	25.06 23.9 26.22
9	17.89 16.6 19.18

Total anthocyanin content (TAC) in the influence of light

Days	Total anthocyanin content (mg/100g)	
	Absence of light	Presence of light
0	48.3 48.7 47.9	48.3 48.7 47.9
3	42.5 44.2 38.4	39.61 37.9 41.32
6	33.8 34.9 38.7	32.48 31.7 33.26
9	32.22 30.84 33.6	26.63 28.23 25.03

Appendix B: Total anthocyanin content (TAC) at different heating temperature mineral content of natural and artificial colors and Antioxidant activity of natural color extracted from pomegranate peel

Total anthocyanin content (TAC) at Heating Temp

Temp	Total anthocyanin content (mg/100g)
Control (Initial value)	48.3 48.7 47.9
30^0	37.31 34.44 30.28
45^0	32.63 32.4 26.26
60^0	29.4 26.85 24.3
75^0	26.54 23.37 20.2

Antioxidant activity of natural color extracted from pomegranate peel

Concentration	% inhibition
0.1	67.32
0.1	66.90
0.1	67.70
0.2	88.58
0.2	87.50
0.2	89.66
0.3	91.76
0.3	89.90
0.3	93.62
0.4	92.79
0.4	90.80
0.4	94.78
0.6	93.16
0.6	91.50
0.6	94.82
0.8	93.63

Appendix C: Mineral content of natural and artificial colors

Mineral content of natural and artificial colors

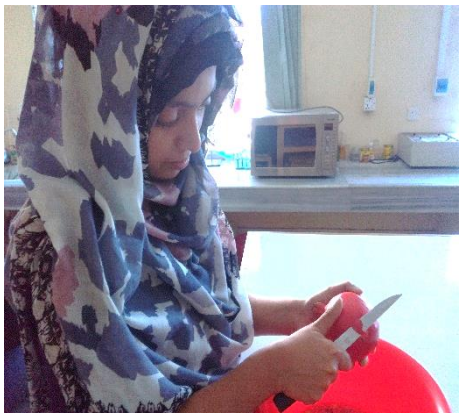
Mineral	Content (mg/100g)	
	N(natural color)	A(artificial color)
Mg	7.2 7.2 7.2	0.2 0.2 0.2
P	20.6 20.6 20.6	0 0 0
Ca	2.15 2.15 2.15	0.39 0.39 0.39
I	1.4 1.4 1.4	0.56 0.56 0.56
K	147.6 147.6 147.6	7.2 7.2 7.2

Appendix D: Sensory Analysis of jelly (Hedonic Rating Test)

Color				Flavor			Texture			Taste			Appearance			General acceptance		
No	SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC
1	9	8	9	9	8	4	8	7	9	9	8	4	8	8	9	9	8	5
2	9	8	7	9	9	6	8	9	6	9	9	8	9	7	9	9	9	9
3	9	9	9	8	9	6	9	9	7	9	9	6	7	7	9	8	9	7
4	8	9	4	9	8	4	8	9	8	9	9	6	9	9	9	8	9	9
5	8	8	7	8	8	7	8	8	7	9	9	7	8	8	8	8	8	7
6	7	7	8	7	8	8	8	7	7	8	8	8	7	8	8	7	7	8
7	9	8	8	8	8	9	9	8	9	9	8	8	8	9	8	9	8	9
8	9	8	9	8	7	8	8	7	7	8	8	6	8	8	9	8	8	8
9	8	7	9	8	7	9	9	7	9	9	7	8	8	8	9	8	7	8
10	8	7	9	8	6	7	7	6	9	7	8	7	8	7	8	8	7	8
11	7	8	8	8	7	6	8	8	6	8	7	5	6	6	8	8	6	6
12	8	7	3	9	6	1	8	8	7	9	8	7	8	6	5	8	6	4
13	8	6	8	6	6	7	8	8	8	8	7	8	8	7	8	8	7	8
14	8	9	9	8	8	9	8	7	9	8	9	7	8	8	9	8	8	7
15	8	8	8	8	8	7	8	7	8	8	8	4	8	7	8	8	7	5

Here, SN= Sample with natural color; SA= Sample with artificial color & SC= commercial product

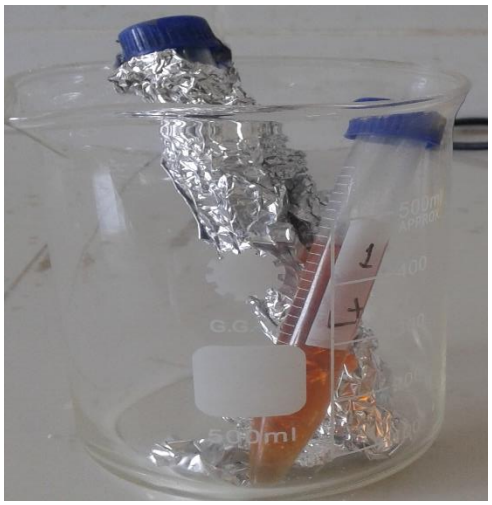
Appendix E: Photo Gallery



Removal of peels

Extraction of color

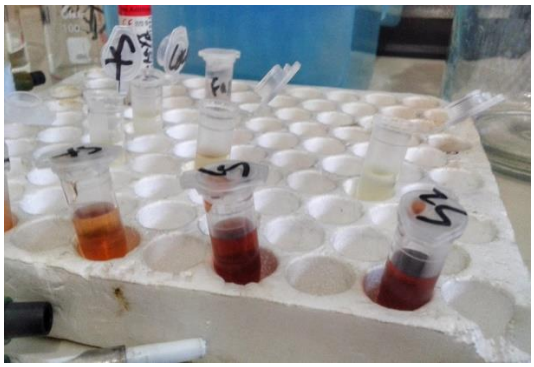
Extracted color



Exposure to light and dark

Heating the color

Analysis of Anthocyanin by UV visible Spectrophotometer



Mineral Analysis

Humalyzer (3000)

Preparation of DPPH solution

Cutting Apple

Pieces of Apple

Artificial color

Jellies with natural (SN) & Artificial Color (SA)

Jelly with natural (SN)

Commercial jelly (SC)

Appendix F: Hedonic Rating Test (Jelly)

Name: **Date:**

Taste this samples and check how much you like or dislike it. Use the appropriate scale to show your attitude by checking at the point that best describe your feelings about the sample, please give a reason for this attitude. **An honest expression of your personal feeling will help us**. Score as follow-

Hedonic	Samples	Attributes																		
		Color			Flavor			Texture			Taste			Appearance			General Acceptance		
		SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC	SN	SA	SC
Like extremely																			
Like very much																			
Like moderately																			
Like slightly																			
Neither like nor dislike																			
Dislike slightly																			
Dislike moderately																			
Dislike very much																			
Dislike extremely																			

Here, SN= Sample with natural color; SA= Sample with artificial color & SC= commercial product

Hedonic Scale Used:
Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, neither like nor dislike = 5, Dislike slightly = 4, Dislike moderately = 3, Dislike very much = 2, Dislike extremely = 1

Brief bio-data of the student

Farhana Sultana passed the Secondary School Certificate Examination in 2008 with GPA 5 followed by Higher Secondary Certificate Examination in 2010 with GPA 4.90. She obtained her B.Sc (Hons.) in Food Science and Technology (BFST) from Faculty of Food Science and Technology, Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh with CGPA 3.64. Now she is a candidate for the degree of MS in Food Processing and Engineering under the Department of Food Processing and Engineering, Faculty of Food Science and Technology, CVASU. She has immense interest to work on Creation and design of Food additives and it's effect on Food Products.