EFFECT OF *IN VITRO* DIGESTION ON THE ANTIOXIDANT CAPACITY OF FIVE INDIGENOUS FRUITS OF BANGLADESH

A THESIS

BY

MAJBAUL ALAM
EXAMINATION ROLL NO.: 1605219
REGISTRATION NO.: 1605219
SESSION: 2016-2017
SEMESTER: JANUARY-JUNE, 2017

MASTER OF SCIENCE
IN
FOOD SCIENCE AND NUTRITION

DEPARTMENT OF FOOD SCIENCE AND NUTRITION
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR

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TO MY

BELOVED PARENTS

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The Author

ABSTRACT

Methanolic extracts of five indigenous fruits (Amla, Elephant apple, Hog plum, Olive and Bilimbi) of Bangladesh were evaluated for biochemical properties, bioactive compound and their antioxidant capacity. Furthermore, the present study also investigated the effect of physiological digestion (*in vitro*) on antioxidant capacity of the studied samples. To improve the knowledge of the antioxidant compound absorption process antioxidant capacity was investigated through ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)), FRAP(Ferric reducing antioxidant power) and DPPH(2,2-diphenyl-1-picrylhydrazyl) assay for undigested and digested extracts. Amla showed the highest value of Vitamin C (523.31mg/100 g FW) and Flavonoid (51.12 mg QE/100 g) in both the case of before and after digestion. On the other hand, maximum value of total phenolics (379.36 mg of GAE/100 g FW) and Flavonol (400.82 µg QE/100g) were found in Elephant apple in both (undigested and digested) condition. All five fruits were significant sources of antioxidants both in terms of total antioxidant capacity and total polyphenols, although results varied significantly ($p<0.05$) among fruits [1.74–12.66 mg $FeSO_4.7H_2O$/ml, 85.67-92.00% inhibition of $DPPH^{\bullet}$, 88.42-91.67% inhibition of $ABTS^{\bullet}$]. Elephant apple revealed maximum level of $DPPH^{\bullet}$ and $ABTS^{\bullet}$ inhibition and Amla displayed highest value for FRAP (mg $FeSO_4.7H_2O$/ml) whereas the other fruits (Hog plum, Bilimbi and Olive) scored lower in the $ABTS^{\bullet}$, $DPPH^{\bullet}$ and FRAP assay at both (digested and undigested) stage. Fruit types and *in vitro* digestion exerted significant ($p<0.05$) effect to all of the indices evaluated.

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ABBREVIATIONS AND ACRONYMS

AA	Ascorbic acid
ABTS	2, 2-azinobis 3-ethyl-benzothiazoline-6-sulfonic acid
ANOVA	Analysis of Variance
AOAD	Antioxidant capacity measured in dichloromethane extract
AOAM	Antioxidant capacity measured in methanol extract
asl	Above sea level
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyl toluene
CAT	Catalase
CUPRAC	Cupric ion reducing antioxidant capacity
DNA	Deoxyribo nucleic acid
DNS	3, 5–di-nitro salicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene di-amine tetra acetic acid
EFSA	European Food Safety Authority
EGCG	Epigallocatechin 3-gallate
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
g	Gram
GAE	Gallic acid equivalent
GI	Gastrointestinal
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S-transferase
HPLC	High Performance Liquid Chromatograhy

HORAC	hydroxyl radical averting capacity
HSTU	Hajee Mohammad Danesh Science and Technology University
IU	International unit
L.s.d	Least significant difference
LDL	Low density lipoprotein
LMWAs	Low molecular weight antioxidants
MDA	Malondialdehyde
M	Mole
mg	Milligram
ml	milliliter
mM	Mill mole
NAC	N-acetyl cysteine
nm	nanometer
ORAC	oxygen radical absorbance capacity
PBS	Phosphate buffer saline
PFRAP	*Polarized Fluorescence Recovery After Photobleaching*
PG	Propyl gallate
QE	Quercetin equivalent
RBS	Reactive Bromine Species
RCS	Reactive chlorine Species
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RS	Reactive species
RSS	Reactive sulfur species
SE	Standard error.
SOD	Superoxide dismutase
SOD	Superoxide dismutase

SPSS	Statistical package for the social sciences
TAC	Total Antioxidant Capacity
TAP	Total antioxidant potential
TBARS	Thiobarbituric acid reactive substances
TBHQ	Terta-butylhydroquinone
TEAC	Trolox equivalent antioxidant capacity
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total flavonoid content
TP	Total polyphenol
TRAP	Total reactive antioxidant potential
UA	Uric acid
UK	United Kingdom
UV-VIS	Ultraviolet and Visible
VCEAC	Vitamin C Equivalent Antioxidant Capacity
w/v	Weight/Volume
WHO	World Health Organization
µl	Micro liter

CHAPTER I

INTRODUCTION

The World Health Organization (WHO) indicates that death from various chronic diseases increases in an alarming way. According to global mortality data reported by the WHO, Chronic disorders caused 38 million deaths in 2009, more than 62% of all deaths worldwide (WHO, 2009). WHO also predicted in a report in 2002 that by 2020 contribution of chronic diseases is expected to rise to 60% of the global burden of diseases and 73% of all deaths. In addition to this, 79% of the deaths due to these diseases will be occurred in developing countries (WHO, 2008). In 2014, WHO reported that in Bangladesh 59% deaths occurred due to various chronic diseases (World Health Organization, 2014).

The main contributor to the pathogenesis of these chronic diseases is free radicals formed in our body due to oxidative stress. The term free radical refers to the reactive oxygen and nitrogen species which are generated in the body by various physiological processes (Gulcin, 2007). During the oxidative stress the commonly formed reactive oxygen species are superoxide ($O_2^{\bullet -}$, $OOH^{\bullet}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals (Aruoma, 1998; Kris-Etherton *et al.,* 2004). Imbalance in the formation of free radicals affect adversely on membrane lipids, proteins, enzymes and DNA which causing oxidative stress and ultimately make the cell death (Gulcin, 2007). This excess degenerative effect providing free radicals formation may be inhibited and abnormal cell damage may be recovered through the action of antioxidants. An antioxidant is a stable molecule which ends the chain reaction prior to the destruction of the vital molecules through donating an electron to the free radical that is lost during oxidative stress (Halliwell, 1995). Antioxidants are usually not synthesized by our body. As a result they must be supplied through the diet. Natural antioxidants in fruits and vegetables provide defensive effects basically by three major groups: phenolics, carotenoids and vitamins. Antioxidants are mainly of two types namely are known as hydrophilic antioxidants (e.g. ascorbic acid and phenolics) and lipophilic antioxidants (e.g. carotenoids) (Halliwell, 1996).

The ingestion of natural antioxidants has shown to enhance the immune defence, reduce risks of cancer, cardiovascular disease, diabetes and other diseases associated with

ageing (Yang *et al.,* 2001 and Sun *et al.,* 2002). WHO (2003) suggested that a daily consumption of at least five servings of fruits and vegetables (equivalent to 400 g) decreases the risk of cardiovascular diseases, chronic diseases and other diseases.

Among the phytochemicals present in plant, phenolic compounds are the main contributor of Antioxidant capacity (Yoo *et al.,* 2008). Due to the ability to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators phenolic compounds are especially important antioxidants (Tsao and Deng, 2004). Due to the complex nature of antioxidants in foods, the study of each individual antioxidant compound is not cost effective. For these reasons, now-a-days, the evaluation of the total antioxidant capacity (TAC) may be an appropriate tool to determine the additive antioxidant properties of plant foods (Pellegrini *et al.,* 2003). Several methods have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables those include 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), (Leong and Shui, 2002), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (Gil *et al.,* 2002), ferric reducing antioxidant power (FRAP) (Guo *et al.,* 2003; Jimenez-Escrig *et al.,* 2001), oxygen radical absorption capacity (ORAC) (Ou *et al.,* 2001; Prior *et al.,* 2003) and so on.

However, the higher time consumption and cost regarding research on human and animal studies show necessity of *in vitro* digestion models which simulate the reactions (chemical and enzymatic) that occur in human digestive system during food digestion. Therefore, at present *in vitro* digestion models gain more attention of the researchers in different food systems (Toor *et al.,* 2009, Bouayed *et al.,* 2011; Cilla *et al.,* 2011; Hur *et al.,* 2011). The amount of nutrients that are available for the absorption after the gastrointestinal digestion is measured by *in vitro* digestion method (Hedren *et al.,* 2002; Kulp *et al.,* 2003). During *in vitro* digestion, foods experienced those conditions similar as they obtains in the gut and the results indicate whether such conditions would lead to release of antioxidants present in the foods or not (Chan *et al.,* 2012).

In the developing countries like Bangladesh where the per capita income is not so high, it is burdensome to bear the cost of the treatment of several chronic diseases. Therefore, increasing the consumption of fruits and vegetables rich in antioxidants could be an effective alternative to prevent these diseases. Thus, research on antioxidant properties of locally available fruits and vegetables could lead to explore their potentiality in dealing with various chronic diseases. Bangladesh is abundant in supply of its typical fruits

which possess functional characteristics such as antioxidant content, cytotoxicity content, antitumor content etc (Zaman, 2015). In the present study, five fruits from five different families has been chosen. These are *Emblica officinalis* (Amla), *Spondias mangifera* (Hog plum), *Dillenia indica* (Elephant apple), *Averrhoa bilimbi* (Bilimbi) and *Elaeocarpus floribundus* (Olive). To the best of our knowledge, the effects of *in vitro* digestion on Antioxidant capacity have not been yet examined in these five indigenous fruits. Therefore, the present study was taken with the following objectives:

a) To analyze biochemical properties of the selected five fruits.
b) To evaluate the bioactive compound content and antioxidant capacity of the fresh extracts of these fruits.
c) To explore the effect of *in vitro* digestion on the antioxidant capacity of these fruits.

CHAPTER II

LITERATURE REVIEWS

2.1 Review on selected fruits

2.1.1 *Emblica officenalis*

General introduction:

Emblica officinalis (amla) is native of tropical India and Southeast Asia, commonly named as 'Indian gooseberry' (Barthakar and Arnold, 1991). Aonla fruits are fleshy, yellowish green in colour having six vague perpendicular furrows enclosing seeds. It belongs to family Euphorbiaceae. It is also named as Amla, Phyllanthus Emblica or Indian Gooseberry (Khan, 2009). Aonla fruit having sour and astringent taste, generally utilized raw, cooked or in the form of pickle (Pant *et al.,* 2004; Mishra *et al.,* 2009).

Aonla fruit is helpful in the treatment of haemorrhage, dysentery, diarrhoea, gastric disorders, constipation, headache, jaundice and enlargement of liver (Parrotta, 2001; Goyal *et al.,* 2007). Various research studies show that aonla has prominent antibiotic, antiulcerogenic, diuretic, laxative, adaptogenic, antitumor, antiscorbutic, hepatoprotective, cardio tonic, antiviral and hypoglycaemic properties (Dahiya and Dhawan, 2001; Pragati *et al.,* 2003; Mishra *et al.,* 2009). Hypolipidaemic effect of fruit juice of aonla was reported in a study by Mathur *et al.* (1996). Study by Perianayagam *et al.* (2004) reported anti-pyretic and analgesic activity in ethanolic and aqueous extract of *Emblica officinalis.*

Nutritional constituents of *Emblica officinalis:*

Aonla has emerged as excellent nutritional source being a rich source of polyphenols and ascorbic acid which are considered to be responsible for their antioxidant properties. Nutritional, commercial and medicinal significance of aonla fruit makes it popular all over the world (Goyal *et al.,* 2007). Aonla is an excellent source of ascorbic acid, amino acid and minerals along with phytochemicals such as polyphenols, tannins, emblicol, linoleic acid, corilagin, phyllemblin and rutin (Jain and Khurdiya, 2004; Murthy and Joshi, 2007; Baliga and Dsouza, 2011).

The nutritional value of Amla (mature, fresh) per 100 grams is as follows:

Table 2.1: Nutritional value of Amla

Nutrients	Percentage (%)
Calories	60
Moisture	81.2 %
Fibre	3.4%
Macronutrient	
Protein	0.4 g
Fat	o.5 g
Carbohydrates	14 g
Micronutrients	
Vitamin B1 (Thiamin)	28 mcg
Vitamin B_3 (niacin)	0.4 mg
Vitamin C	720 mg
Nicotinic Acid	0.2 mg
Calcium	15 mg
Iron	1 mg
Phosphorous	21 mg

(Source: Zulkharnain, 1996)

2.1.2 *Spondias mangifera*

General introduction:

Hog plum (*Spondias manigfera*) belongs to the Anacardiaceae family, native to the South America, Africa, Indonesia, Sri Lanka, India and Bangladesh as a summer fruit (Islam *et al.*, 2015). It is a deciduous, glabrous tree grows up to 25 m in height and grows at an altitude of 550 m asl(above sea level) to 1500 m asl. All parts of the plant emit a fetid, turpentine like odour when broken or brushed; the smell varies from species to species (Satpathy *et al.*, 2011). Leaves are compound with the leaflets base often asymmetric. Inflorescence is axillary, flowers are white to cream, polygamous. Fruit is a drupe with a large stone, ellipsoid to elliptic-ovoid, yellowish orange at maturity, 3.5-5 × 2.5-3.5 cm Inner part of endocarp is woody and grooved, outer part is fibrous (Andola and Purohit, 2010; Longman, 2005). The unripe fruits are sour, thermogenic, appetizer and aphrodisiac, while ripe ones are sweet–sour with a hard stone.

Nutritional constituents of *Spondias mangifera:*

Andola and Purohit (2010) observed that the ripe fruits of *Spondias mangifera* from two location (Chauki and Maletha) of Western Himalaya contain moisture (71.33±1.53 and

76.00±1%), ash (4.92±0.04 and 4.30±0.05%), crude fiber (4.03±0.05 and 3.13±0.12), fat (12.23±0.06 and 12.54±0.04%), crude protein (2.12±0.05 and 0.87±0.04%), total carbohydrate (23.54±0.50 and 16.30±0.05%), food energy value (189.55±0.44 and 203.52±1.00 kcal/g), nitrogen (0.34±0.02 and 0.14±0.02%), phosphorus (0.68±0.01 and 0.67±0.02%), potassium (1.38±0.80 and 0.96±0.01%), sodium (1.54±0.01 and 0.12±0.03%), calcium (0.93 and 0.15±0.5%), iron (1.32±0.02 and 1.25±0.04%) and copper (1.23±0.03 and 0.9±0.01%) respectively.

Sivaprasad *et al.* (2011) observed that mature *Spondias mangifera* willd. contain spondiol [(22.25 mg), total phenolics (25-30 mg), total carotenoids (300 mg), total chlorophyll (1500 mg), reducing sugar (0.04 mg), total sugar (3.6 mg), ascorbic acid (22.5 mg)]/100g fresh weight, titrable acidity (4.25%), pH (2.54), TSS (7.15obrix) and total protein (17 mg/100g fresh weight).

2.1.3 *Dillenia indica*

General introduction:

Dillenia indica, commonly known as elephant apple is a wildly and widely available fruit in the north eastern states of India and foothills of Himalaya (Abdille *et al.*, 2005). Dillenia indica (D. indica) plant belongs to family Dilleniaceae, commonly called Dillenia. The fruit shows laxative properties and is used for relieving abdominal pain. The bark and leaves have astringent effect (Kritikar and Basu, 2003). This evergreen shrub is widely distributed in India, Indonesia, Thailand, China and other places, producing ripe sweet-sour fruits as a nutritious food which is eaten fresh or cooked, or made into jams and jellies although the unripe fruit is acid, astringent and seldom used as food. Traditionally, the fruits, leaves and barks of *D. indica* are used to treat the diseases such as fever, constipation, diarrhea, stomach pain and so on (Yazan and Armania 2014). The fruit decoction had been adopted to treat hair loss and diabetes and was also an immunity enhancer (Gandhi and Mehta 2014). Juice of elephant apple mixed with sugar and water can function as a cough syrup with cooling effect (Yazan and Armania, 2014). Extracts of phytochemicals from *D. indica* possess several biological activities such as anti-inflammatory, antioxidant, antidiabetic, anticholinesterase, antimicrobial and cytotoxic activities (Kumar *et al.*, 2010; Kumar and *et al*., 2011; Migliato *et al*., 2011; Bhadra *et al*., 2014). Different extracts of *D. indica* fruits showed different antileukemic

activity against human leukemia cell lines, there into, the ethyl acetate extracts gave the highest cytotoxic activities (Kumar *et al*., 2010).

Nutritional constituents of *Dillenia indica*:

The phytochemical constituent of *Dillenia indica* Linn. Crude extract include Glycoside, Steroids, Flavonoids, Saponines and reducing sugar. The investigation of the phytochemical constituent indicates that the leaves are provided a rich source of triterpenoids and flavonoids. It also reported to contain various chemical constituents like 3, 5, 7-trihydroxy-3', 4'-dimethoxy flavone (dillenetin), betulinic acid, β-sitosterol and stigmasterol (Abdul *et al.,* 2010).

Chief contents of the fleshy sepals are tannins, malic acid, arabinogalactan and glucose. They also contain an arabinogalactan, betulin, betulinic acid and flavonoids. Bark and wood contain flavonoids, betulin, betulinic acid, betulinaldehyde, lupeol, β-sitosterol, myricetinhydroxy-lactone, dihydroisorhamneti, dillentin and glucosides. Stem bark contains betulinaldehyde, betulin, lupeol, β-sitosterol, myricetin, a new hydroxylactone, dihydro-isorrhamnetin, dillentin and glucosides (Bose *et al.,* 2010)**.**

Table 2.2: Nutritional value of Elephant Apple

Nutrients	Percentage (%)
Calories	59
Moisture	76.2 %
Fibre	2.1- 2.5%
Macronutrient	
Protein	0.4 g
Fat	0.2- 0.34 g
Ash	
Carbohydrates	14 g
Micronutrients	
Vitamin C	720 mg
Calcium	16 mg
Phosphorous	26 mg

(Source: Saikia and Dutta, 1995)

2.1.4 *Averrhoa bilimbi*:

Averrhoa bilimbi (Oxalidaceae family) commonly known as bilimbi, is an attractive, long-lived tropical tree, reaching 5-10 m in height; has a short trunk soon dividing into a number of upright branches. Fruit ellipsoid, obovoid or nearly cylindrical, faintly 5-sided, 4-10 cm long; capped by a thin, star-shaped calyx at the stem- end and tipped with 5 hair like floral remnants at the apex. Crispy when unripe, the fruit turns from bright green to yellowish-green, ivory or nearly white when ripe and falls to the ground. The outer skin is glossy, very thin, soft and tender and the flesh green, jelly like, juicy and extremely acid. There may be a few 6-7 flattened, disc-like seeds, 6 mm wide, smooth, brown (Kumar *et al.*, 2011).

These Averrhoa bilimbi easily found in most country like Indonesia, Malaysia, Brazil, Cuba, Philippines and Sri Lanka as well as in Bangladesh and Myanmar (Burma). It used for treatment of children cough, stomach, ache and as a cooling drink. On the other hand, A. bilimbi, has been widely used in traditional medicine for treatment of cough, rheumatism, itches, boils, diabetes, syphilis, whooping cough, cold and hypertension (Goh *et al.*, 1995).

Nutritional constituents of Bilimbi:

Bilimbi is a nutrition-packed, starchy fruit that grows mostly on the trunk of tall trees. It is a rich source of Vitamin C. Other than the vitamins and minerals, the fruit also consists of fibre, ash, protein and moisture as well as minerals.

Table 2.3: Nutritional value of Bilimbi

Nutrients	Percentage (%) or per 100 gm
Moisture	94.2-94.7%
Protein	0.61%
Fiber	0.6%
Ash	0.31-0.40%
Ascorbic Acid	15.5mg
Vitamin B1 (thiamine)	0.010 mg
Riboflavin	0.302mg
Niacin	0.302mg
Calcium	3.4mg
Phosphorus	11.1mg
Iron	1.01mg

(Source: Zakaria *et al.*, 2007)

2.1.5 *Elaeocarpus floribundus*

The olive fruit (*Elaeocarpus floribundus*) is a small , thinner- fleshed drupe that is 1-1.25 cm long and grows on a small tree, which belongs to the family Elaeocarpaceae *and* is native to tropical and warm temperate regions of the world (Perez *et al.*, 2005).The olive has high oil content and extracted olive oil is very popular for its nutritive and health promoting potential, especially against cardiovascular disorders due to the presence of high levels of mono-unsaturated fatty acids and flavonoid polyphenols such as hydroxytyrosol and tyrosol. These components are known to possess various biological activities such as antioxidant, anticarcinogenic, anti- inflammatory, antimicrobial, anticarcinogenic, anti- inflammatory, antimicrobial, antihypertensive, antidyslipidemic, cardiotonic, laxative and antiplatelet. Naturally, olive fruits have a bitter flavor; hence they are subjected to fermentation or cured with lye or brine to make them more palatable (Tuna and Bayizit, 2009).

Nutritional constituents of *Elaeocarpus floribundus*:

Olive fruit contains high amount of phenolic compounds. Oleuropein is the main phenolic compound existing in the pulp fraction of the olive fruit (Omar 2010). The average composition of olive fruit is difficult to define due to its remarkable diversity, producing high compositional variability. However, the water and fat are indubitably the main constituents, beside other water- soluble compounds (sugars, organic acids, nitrogenous compounds, phenols) and insoluble fraction of colloids such as hemicelluloses, celluloses, pectins, enzymatic and structural proteins (Servili *et al.*, 2012).

Table 2.4: Nutritional value of Olive

Nutrients	Percentage (%) or per 100 gm
Water	50-60
Oil	15-30
N- matter	2-5
Sugars	3-75
Cellulose	3-6
Ash	1-2
Phenols	2-2.5

(Source: Ryan *et al.*, 1999)

2.2 Free radicals

Free radicals are any molecule containing an unpaired electron (Milan *et al*., 1998). These free radicals are unbalanced molecules that steal electron from healthy cells, causing a chain reaction. When the chain reaction occurs, it can cause cell damage or cell death. The major sources of radicals in human body are leakages from the electron transport chain of mitochondria.

The oxygen utilization by mitochondria of aerobic organisms can generate several reactive radicals, such as Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), Reactive chlorine Species (RCS) and Reactive Bromine Species (RBS) (Pollack & Leeuwenburgh, 1999). Reactive species (RS) cause oxidative damage of biomolecules that increase with age and is postulated to be a major factor of degenerative diseases and biochemical senescence.

Free radicals may be formed by any one of the following ways:

1) by the hemolytic cleavage of a covalent bond of a normal molecule (each fragment retaining one of the paired electrons)
2) by the loss of a single electron from a normal molecule
3) by the addition of a single electron to a normal molecule

Free radicals can be positively charged, negatively charged or electrically neutral. Some examples of reactive species are shown in Table 2.5.

Table 2.5: Reactive species

Free radicals	Non-radicals
Reactive oxygen species (ROS)	Hydrogen peroxide (H_2O_2)
Superoxide ($O_2^{\bullet-}$)	Hypobromous acid (HOBr)
Hydroxyl ($OH^{\bullet}$)	Hypochlorous acid (HOCl)
Reactive oxygen species (ROS)	Organic peroxides (ROOH)
Hydroperoxyl ($HO_2^{\bullet}$)	Ozone (O_3)
Carbonate ($CO_3^{\bullet-}$)	Singlet oxygen ($^1O_2$)
Peroxyl ($RO_2^{\bullet}$)	Peroxynitrite ($ONOO^-$)
Alkoxyl ($RO^{\bullet}$)	Peroxylnitrate (O_2NOO^-)
Carbon dioxide ($CO_2^{\bullet-}$)	Peroxynitrous acid ($ONOOH^-$)
Single ($^1O_2$)	Nitrosoperoxycarbonate ($ONOOCO_2^-$)
Reactive chlorine species (RCS)	Hypochlorous acid (HOCl)
Altomic chlorine ($CL^{\bullet}$)	Chloramines
Reactive bromine species (RBS)	Bromine chloride (BrCl)
Atomic bromine ($Br^{\bullet}$)	Bromine chloride (BrCl)
Reactive nitrogen species (RNS)	Chlorine gas (Cl_2)
Nitric oxide ($NO^{\bullet}$)	Bromine gas (Br_2)
Nitogen dioxide ($NO_2^{\bullet}$)	Chlorine dioxide (ClO_2)
Nitrate ($NO_3^{\bullet}$)	Alkyl peroxynitrites (ROONO)
	Peroxynitrite ($ONOO^-$)
	Dinitrogen trioxide (N_2O_3)
	Dinitrogen tetroxide (N_2O_4)
	Nitrosyl cation (NO^+)
	Nitroxyl anion (NO^-)

(Source: Changpraykal, 2016)

2.3 Antioxidant

The first definition of antioxidant was proposed by Halliwell in 1989 as "any substance that, present in low concentrations compared to oxidizable substrates (carbohydrates, lipids, proteins or nucleic acids), significantly delays or inhibits the oxidation of the mentioned substrates" (Halliwell, 1992). Later, other definitions of antioxidant were proposed, such as "any substance that prevents, delays or eliminates oxidative damage of a target molecule" (Halliwell and Gutteridge, 1990) or "any substance that can eliminate reactive oxygen species directly or indirectly, acting as a regulator of the antioxidant defense, or inhibiting the production of those species" (Khlebnikov *et al.,* 2007).

Reactive oxygen species (ROS) are a group of molecules produced by some metabolic processes, due to the action of oxidases in the mitochondria or other cellular compartments. ROS have high reactivity because they possess unpaired electrons that can interact with oxidizable substrates through redox reactions. The main ROS involved in the biological systems are superoxide anion, hydroxyl radical, hydroperoxyl and peroxyl radical, nitric oxide and other species such as hydrogen peroxide, singlet oxygen and hypochlorous acid (Fransen *et al.*, 2012). However, there are other reactive molecules derived from the reaction of ROS with nitric oxide (reactive nitrogen species, RNS) or thiols (reactive sulfur species, RSS) (Corpas and Barroso, 2015) (Figure 2.1).

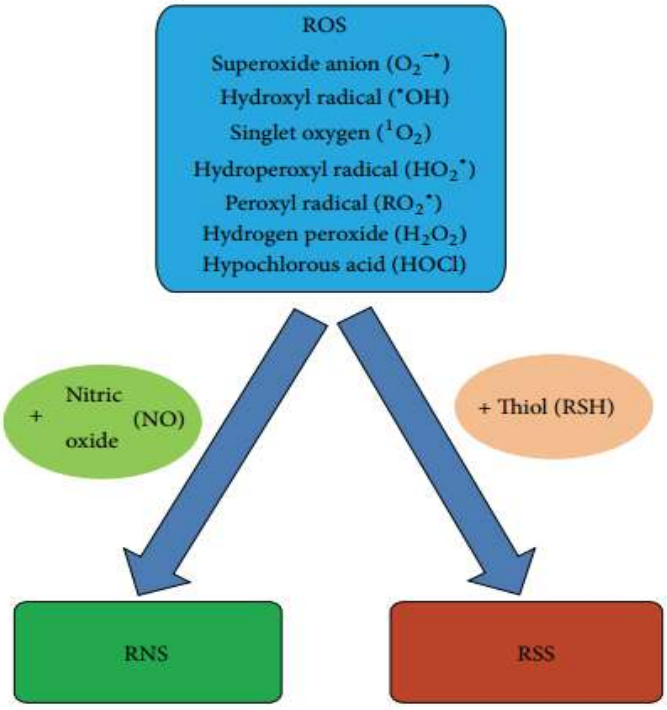

Figure 2.1: Reactive Oxygen species (ROS) and derivatives

The balance between oxidants and antioxidants (redox balance) is essential in maintaining a healthy cellular microenvironment. The generation of oxidative stress is caused by an alteration in the balance between ROS production and the efficiency of the cell antioxidant defense system. Cells and tissues are continuously being exposed to free radicals derived from the metabolism or external factors, such as pollution, microbes, allergens, radiation, cigarette smoke and pesticides (Hekimi *et al.*, 2011). However, ROS can play a dual role, acting as beneficial or harmful factors (Kawagishi and Finkel, 2014). On the one hand, the increase in ROS production generates oxidative stress, a damaging process that can alter cell structures and influences the expression of genes

related to accelerated cell aging (Popa-Wagner, 2013). Nevertheless, ROS derived from the mitochondrial respiratory chain, at low or moderate concentrations, participate in physiological functions such as in the defense against infections and in the maintenance of redox (Zhao, 2009). Cells have several mechanisms to transform and eliminate ROS to avoid their harmful effects. The synergistic action of both antioxidant proteins and enzymes and exogenous antioxidants neutralize free radicals and modulate cell signaling (Watson *et al.,* 2011). In fact, numerous studies suggest that antioxidants exert a protective effect against radiation and also prevent the development of many diseases such as cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration and diabetes (Fang *et al.,* 2002).

2.3.1 The Antioxidant Defense

The natural antioxidant defense is composed of endogenous antioxidants, which are enzymatic and non-enzymatic antioxidants produced by our own body and exogenous antioxidants, which can be incorporated through the diet or nutritional supplements (Goodman *et al.,* 2011). Furthermore, there is another group that comprises synthetic antioxidants widely used in the food industry, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and terta-butylhydroquinone (TBHQ). Several in vivo studies carried out in the 80s and the 90s reported some health risks associated with the consumption of synthetic antioxidants (Goodman *et al.,* 2011).However, this is a controversial issue. A trial conducted in 1993 suggested that the toxic effects produced by BHA and BHT occur only at high doses in long-term treatments (Kahl and Kappus, 1993). Another study found that the usual intake of BHA and BHT at low doses is not associated with stomach cancer risk (Botterweck *et al.,* 2000). More recently, the European Food Safety Authority (EFSA) studied in depth all the contradictory published data and established that the acceptable daily intakes of 0.25 mg/kg/day for BHA and 1.0 mg/kg/day for BHT are safe for adults and children (Carocho and Ferreira, 2013).

Antioxidants can be classified into three lines of defense according to their mechanism of action. The first line includes antioxidants that prevent the formation of new free radicals. It is a very heterogeneous group which includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX); proteins that bind metals such as ferritin and ceruloplasmin; and minerals such as Se, Cu and Zn. The

second group of antioxidants is responsible for capturing free radicals and thus they prevent oxidative chain reactions. This group is formed by the glutathione enzyme, albumin, vitamins C and E, carotenoids and flavonoids. The third line of defense includes antioxidant enzymes that repair the damage caused by free radicals to biomolecules, such as lipases, proteases, DNA repair enzymes, transferases and methionine-sulfoxide reductases (Shetti *et al.,* 2009; Sindhi *et al.,* 2013) . Most exogenous antioxidants are produced by vegetables. Therefore, they are often called phytochemicals, although this is a concept which refers to any chemical compound derived from plants (Gharras, 2009).

Exogenous antioxidants constitute a very large and diverse group of molecules in terms of chemical structure and biological properties (Landete, 2012). Due to the abundance and diversity of members, this group can be divided into three subgroups: polyphenols, vitamins and derivatives and antioxidant minerals (Carocho and Ferreira, 2013). Polyphenols are the most abundant natural antioxidants. The two main types of polyphenols are flavonoids and phenolic acids. For its part, flavonoids can be classified into several groups: flavonols, flavanones, flavones, catechins, anthocyanins and isoflavones. Polyphenols are usually secondary metabolites involved in the defense against UV radiation or pathogens (Quideau *et al.,* 2013). They are found in all plant products such as fruits, vegetables, juices, tea and wine and they contribute to their color, taste, smell and oxidative stability (Graf *et al.,* 2005). Numerous epidemiological studies in the late twentieth century have suggested that polyphenols confer some protection against the development of prevalent diseases, including diabetes, infections, cancer, cardiovascular diseases, asthma and osteoporosis (Landete, 2012).

Within the family of vitamins and derivatives, highlighted members are vitamins: C, E and K and carotenoids. Carotenoids are a group of pigments present in many fruits and vegetables. There are more than 600 types, but only a few of them have demonstrated biological properties, as is the case of β-carotene and lycopene. β-Carotene is the most studied antioxidants for the prevention of diseases (Bennett *et al.,* 2012). A product of the hepatic catabolism of β-carotene is vitamin A or retinol, which has beneficial effects on the skin, eyes and internal organs and that has the ability to combine and neutralize peroxyl radicals before they produce lipid peroxidation (Perez-Rodrıguez, 2010; Rao and Rao, 2007).

Vitamin C or ascorbic acid is known by its electron donating ability, thanks to which it prevents the accumulation of oxidizing agents and free radicals. It is especially efficient in eliminating superoxide anion radicals, hydrogen peroxide, hydroxyl, singlet oxygen and RNS (Barros *et al.,* 2011; Traber and Stevens, 2011).

Within the group of antioxidant minerals, selenium has a special importance because it is a cofactor of antioxidant enzymes such as GP Xandthioredoxin reductase, among others (Puspitasari *et al.*, 2014).

2.3.2 Classification of Antioxidants

Antioxidants can be categorized in multiple ways. Based on their activity, they can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids and glutathione (Shahidi and Zhong, 2010).

The other way of categorizing the antioxidants is based on their solubility in the water or lipids. The antioxidants can be categorized as water-soluble and lipid-soluble antioxidants. The water-soluble antioxidants (e.g. vitamin C) are present in the cellular fluids such as cytosol, or cytoplasmic matrix. The lipid-soluble antioxidants (e.g. vitamin E, carotenoids and lipoic acid) are predominantly located in cell membranes.

The antioxidants can also be categorized according to their size, the small-molecule antioxidants and large-molecule antioxidants. The small-molecule antioxidants neutralize the ROS in a process called radical scavenging and carry them away. The main antioxidants in this category are vitamin C, vitamin E, carotenoids and glutathione (GSH). The large-molecule antioxidants are enzymes (SOD, CAT and GSHPx) and sacrifcial proteins (albumin) that absorb ROS and prevent them from attacking other essential proteins (Nimse and Dilipkumar, 2015).

2.3.3 Health Benefits of Antioxidants

The benefit of antioxidant uptake has been demonstrated in the course of some diseases and certain conditions as diabetes, asthma, hemodialysis, thalassemia, rheumatoid arthritis, systemic attack, post menopause, schizophrenia, depression and leukemia (Mushtaq and Wani, 2013; Kandasamy and Ashokkumar; 2014) (Figure 2.2).

The consumption of polyphenols has been associated with the prevention of the development of atheromatous lesions (Maeda *et al.,* 2003), the reduction of the size of such lesions in vivo (Miura *et al.,* 2001) and the inhibition of platelet aggregation *in vitro* (Russo, 2001) and in vivo (Bertelli and Das, 2009). In addition, polyphenols seem to reduce the oxidation of LDL, a process that may be responsible for atherosclerosis development. For its part, tea catechins inhibit proliferation and invasiveness of smooth muscle cells in the artery walls of experimental animals. This effect could contribute to reducing the formation of atheromatous lesions. However, this effect has not been fully clarified in humans (Maeda *et al.,* 2003).

Several studies suggested that the consumption of foods rich in polyphenols can prevent the development of these diseases (Letenneur, 2007; Ebrahimi and Schluesener, 2012). Green tea provides protection against Parkinson (Zhao, 2009) and daily consumption of wine has been linked to a lower incidence of dementia and Alzheimer (Swaminathan and Jicha, 2014). In fact, it has been shown that dietary polyphenols act against hydrogen peroxide, being more effective than vitamins (Dai *et al.,* 2006). Similarly, the consumption of fruit and vegetable juices may also play an important role in delaying the development of neurodegenerative disease (Vingtdeux *et al.,* 2008).

It is believed that antioxidants can prevent the development of cancer due to their effects on cell cycle regulation, inflammation, the inhibition of tumor cell proliferation and invasiveness, the induction of apoptosis and the stimulation of the detoxifying enzyme activity (Valko *et al.,* 2007). The antitumor effect of some polyphenols, such as catechins, isoflavones, lignans, flavanones, resveratrol, ellagic acid, quercetin and curcumin, has been extensively studied. It has been found that these compounds are able to reduce tumor growth through various action mechanisms, in different locations such as mouth, stomach, liver, lung, duodenum, colon, mammary gland and skin (Hu *et al.,* 2011; Lu *et al.,* 2011).

One important antioxidant is resveratrol, since it has demonstrated both in vivo and *in vitro* ability to slow down tumor progression in experimental models of lung, skin, breast and colon cancer, it interferes with the inflammatory mechanisms and it has antiangiogenic and antimetastatic properties (Lu *et al.*, 2011; Vaz-da-Silva *et al.*, 2008). These findings, coupled with the fact that high doses of oral resveratrol seem to be nontoxic, make resveratrol a promising antioxidant for cancer therapy (Juan *et al.*, 2002).

However, some studies have shown reported benefits of consuming antioxidant supplements, such as the trial made by Lappe *et al. (*2007) which showed that supplementation with vitamin D and calcium could reduce the overall risk of cancer in postmenopausal women older than 55 years.

A study conducted in Bangladesh, which began in 2006, was to prove the administration of vitamin E and selenium for five years, individually and in combination, to offset the adverse effects of exposure to arsenic suffered by the population. However, they found that although the treatment improved the evolution of lesions, there was an increase in mortality and skin dysplasia in the supplemented patients (Verret *et al.*, 2005).

Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy and longevity (Kalcher *et al.*, 2009). Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes (Granero *et al.*, 2008; Cummings *et al.*, 2001). The recommendations based on epidemiological studies are such, that fruits, vegetables and less processed staple foods ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract (Halvorsen *et al.*, 2002). The explanation consists in the beneficial health effect, due to antioxidants present in fruit and vegetables (Halvorsen *et al.*, 2006).

Fruit juices, beverages and hot drinks contain high amounts of antioxidants, like polyphenols, vitamin C, vitamin E, Maillard reaction products, β-carotene and lycopene (Ramadan-Hassanien, 2008). The consumption of fruit juices, beverages and hot drinks was found to reduce the morbidity and mortality caused by degenerative diseases (Quirós and Costa, 2006).

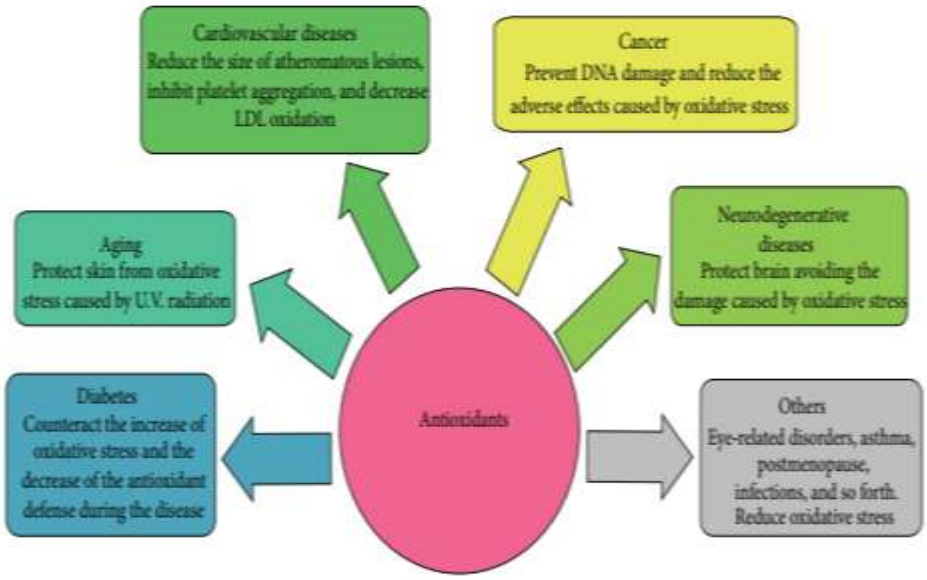

Figure 2.2: Influence of antioxidants on human health.

(Sources: Mushtaq and Wani, 2013 and Kandasamy and Ashokkumar, 2014)

2.3.4 Methods of Total Antioxidant Capacity Assessment

The various analytical methods (Giardi *et al.*, 2010) of evaluation of the antioxidant capacity fall into distinct categories:

Antioxidant capacity assay	Principle of the method	End-product determination
	Spectrometry	
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
PFRAP	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe3+	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC	Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein
HORAC	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein

TRAP	Antioxidant capacity to scavenge luminol-derived radicals, generated from AAPH decomposition	Chemiluminescence quenching
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/emission spectra
	Electrochemical Techniques	
Cyclic voltammetry	The potential of a working electrode is linearly varied from an initial value to a final value and back and the respective current intensity is recorded	Measurement of the intensity of the cathodic/ anodic peak
Amperometry	The potential of the working electrode is set at a fixed value with respect to a reference electrode	Measurement of the intensity of the current generated by the oxidation/reduction of an electroactive analyte
Antioxidant capacity assay	**Principle of the method**	**End-product determination**
Biamperometry	The reaction of the analyte (antioxidant) with the oxidized form of a reversible indicating redox couple	Measurement of the current flowing between two identical working electrodes, at a small potential difference and immersed in a solution containing the analysed sample and a reversible redox couple
	Chromatography	
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionisation or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at high flow rate and pressure of the mobile phase	UV-VIS (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

2.3.5 Antioxidant Capacity of Fruits

Thaipong *et al.* (2006) reported on estimating antioxidant capacity from guava fruit extracts. Guava fruit extracts were analyzed for antioxidant capacity measured in methanol extract (AOAM), antioxidant capacity measured in dichloromethane extract (AOAD), ascorbic acid, total phenolics and total carotenoids contents. The ABTS, DPPH and FRAP assays were used for determining both AOAM and AOAD, whereas the ORAC was used for determining only AOAM. Averaged AOAM [mM Trolox equivalent (TE)/g fresh mass (FM)] were 31.1, 25.2, 26.1 and 21.3 as determined by the ABTS, DPPH, FRAP and ORAC assays, respectively. Averaged AOAD (mM TE/g FM) were 0.44, 0.27 and 0.16 as determined by the ABTS, DPPH and FRAP assays, respectively.

Shukla (2009) evaluated the antioxidant profile and activity of amalaki (*emblica officinalis)*, spirulina and wheat grass aqueous and alcoholic extracts of amlaki (Emblica officinalis), spirulina and wheatgrass were prepared and analyzed for antioxidant vitamin content (vitamin C and E), total phenolic compounds. Antioxidant status, reducing power and effect on glutathione S-transferase (GST) activity were evaluated *in vitro*. Vitamin C content of crude amlaki powder was found to be 5.38 mg/g, while very less amount 0.22 mg/g was detected in wheat grass. Total antioxidant capacity of aqueous extract of amlaki, spirulina and wheat grass at 1mg /ml concentration were 7.78, 1.33 and 0.278 mmol/l respectively. At similar concentrations the total antioxidant capacity of alcoholic extract of amlaki, spirulina and wheat grass was 6.67, 1.73 and 0.380 mmol/l respectively. Amlaki was also found to be rich source of phenolic compounds (241mg/g gallic acid equivalent). Alcoholic extract of wheat grass showed 50 % inhibition in $FeCl_2$- ascorbic acid induced lipid peroxidation of rat liver homogenates *in vitro*. Both aqueous and alcoholic extracts of amalaki inhibited activity of rat liver glutathione S-transferase (GST) *in vitro* in dose dependant manner.

Nurul and Asmah (2012) examined on antioxidant properties in fresh and pickled papaya. This study compared total phenolic (TPC), total flavonoid (TFC), β-carotene, lycopene, ascorbic acid (AA) contents and antioxidant properties between fresh and pickled papaya. The results indicated that mean TPC (mg gallic acid equivalent/100 g dry samples), TFC (mg rutin equivalent/100 g dry samples), β-carotene (μg/100 g edible portions), lycopene (μg/100 g edible portions) and AA content (mg/100 g edible portions) were higher in fresh papaya (141.66 ± 11.71; 57.80 ± 2.11; 793.83 ± 5.47; 779.69 ± 5.55; 70.37 ± 0.65) as compared to pickled form. Antioxidant capacity (%)

measured by DPPH and β-Carotene-Linoleate bleaching method was higher in fresh papaya (56.83 ± 4.68; 77.56 ± 1.40). Total phenolic, total flavonoid, ascorbic acid, beta carotene and lycopene were strongly correlated with Antioxidant capacity and scavenging activity ($0.905 \leq r \leq 1.00$) indicating that were important contributors to antioxidant properties in papaya extracts. The pickling process of papaya caused a significant decrease in their antioxidant component and activity.

Dorta *et al.* (2012) analyzed the effect of different drying treatments on the Antioxidant capacity of mango peel and seed. Oven-drying at 70 ^{0}C (with static or forced air) was the treatment that had the most negative effect on the antioxidant capacity of mango peel (when extraction was carried out with ethanol) and seed. Because the effect of drying methods on the phenol and anthocyanin content of mango peel or seed is related to antioxidant capacity (with a moderately strong significant correlation, $p < 0.003$), it can be concluded that the phenol content of both materials is largely responsible for their antioxidant capacity.

Nisco *et al.* (2013) showed nutraceutical properties and polyphenolic profile of berry skin and wine of *Vitis vinifera*. Red grapes are rich in phenolics, flavonoids, anthocyanins and resveratrol, all substances which have been suggested as having nutraceutical and health benefits. HPLC analysis coupled with LC–ESI/MS/MS detected high contents of total flavonols and anthocyanins in berry skin and wine. The wine made with the same grape used for berry skin assays showed a notable presence of quercetin-3-O-glucoside (39.4% of total flavonols) and malvidin and petunidin derivatives (63.9% and 10.8% of total anthocyanins, respectively). The strong antioxidant ROS-scavenging activity, determined by both DPPH and FRAP assays and the high resveratrol content confer high sensory characteristics resulted to be associated with positive nutraceutical properties of these grapes and wine. The level of cis-resveratrol was lower than trans-resveratrol in both berry skin and wine reaching 44.1 mg/kg and 0.3 mg/l, respectively.

Almeida *et al.* (2011) reported on bioactive compounds and antioxidant capacity of fresh exotic fruits from northeastern brazil. The antioxidant activities were evaluated using two antioxidant systems 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), expressed as TEAC (Trolox Equivalent Antioxidant Capacity) and VCEAC (Vitamin C Equivalent Antioxidant Capacity) values. The results indicated that the above fruits, such as murici and mangaba, were

good sources of antioxidants. The phenolic contents showed positive correlations with total antioxidant by ABTS ($R=0.94$, $P\leq0.001$) and DPPH ($R=0.88$, $P\leq0.001$) assays.

2.4 *In vitro* digestion model

In vitro (*meaning*: in the glass) studies are conducted using components of an organism that have been isolated from their usual biological surroundings, such as microorganisms, cells, or biological molecules. *In vitro* digestion models are widely used to study the structural changes, digestibility and release of food components under simulated gastrointestinal conditions. In the past few years, there has been an increasing interest in the structural design of food-based delivery systems to encapsulate, protect and release bioactive components believed to benefit human health (McClements *et al.*, 2009). These delivery systems may be designed to release the bioactive components at a specific location in the human gastrointestinal (GI) tract, often in response to an environmental trigger, such as pH, ionic strength, or enzyme activity.

In vivo feeding methods, using animals or humans, usually provide the most accurate results, but they are time consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures (Boisen & Eggum, 1991). In principle, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients. The ideal *in vitro* digestion method would provide accurate results in a short time (Coles *et al.*, 2005) and could thus serve as a tool for rapid screening foods or delivery systems with different compositions and structures. In practice, any *in vitro* method is inevitably going to fail to match the accuracy that can be achieved by actually studying a food in vivo due to the inherent complexity of the process (Coles *et al.*, 2005). Consequently, some compromise is needed between accuracy and ease of utilization of any *in vitro* digestion model. During the past few years, food and animal scientists have utilized a number of *in vitro* digestion models to test the structural and chemical changes that occur in different foods under simulated GI conditions, although none of these methods has yet been widely accepted.

The *in vitro* digestion models may differ from one another in their operation. According to Hur *et al.*, 2011 those include:

(1) The number and type of steps included in the digestion sequence, e.g., mouth, stomach, small intestine, large intestine.

(2) The composition of the digestive fluids used in each step, e.g., enzymes, salts, buffers, biological polymers and surface-active components.

(3) The mechanical stresses and fluid flows utilized in each step in the digestion sequence, e.g., magnitude and direction of applied stresses, flow geometries and flow profiles.

In addition, there are considerable differences in the type of experimental parameters measured in the various digestion models. These include chemical changes (such as hydrolysis of lipids, proteins and/or polysaccharides), location changes (such as release of encapsulated components, competitive adsorption processes, multilayer formation) and structural changes (such as breakdown of specific structures, aggregation, droplet coalescence, or droplet disruption).

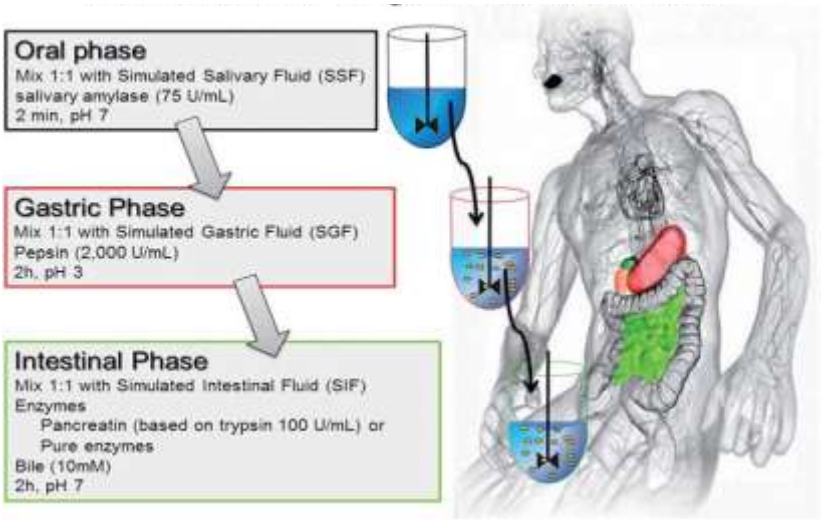

Figure 2.3: *In vitro* digestion method for food matrix

2.4.1 *In vitro* digestion and enzymes

Several factors, such as sample characteristics, enzyme activity, ionic composition, applied mechanical stresses and digestion times, have significant influences on the results of *in vitro* digestion methods. Therefore, *in vivo* conditions can never be completely simulated under *in vitro* conditions (Boisen & Eggum, 1991). An early study by Boisen and Eggum (1991) defined the relationship between *in vitro* digestion and enzyme activity. They reported that the *in vitro* technique can be designed to use specific enzymes either to give maximal digestibility values or to measure the initial rate of

hydrolysis. The most important factor in an *in vitro* digestion system is the enzyme characteristics. Several factors, such as concentration, temperature, pH, stability, activators, inhibitors and incubation time, affect enzyme activities. The choice of enzymes and incubation conditions and the need for equipment are also dependent on the objectives of the study (Boisen & Eggum, 1991). Single-enzyme methods can be useful for predicting the digestibility of single nutrients, e.g., protein by the use of pepsin, starch by the use of amylase, or lipids by the use of lipases (Boisen & Eggum, 1991). It has been reported that using a single purified enzyme, rather than a complex biological mixture, is often advantageous because it facilitates the standardization of *in vitro* digestion models, which enables more consistent laboratory-to-laboratory comparisons (Coles *et al.,* 2005). However, the digestion of one nutrient is often influenced by the digestion of other nutrients and so it is often more realistic to use a complex mixture of enzymes rather than a single purified one (Boisen & Eggum, 1991).

The most frequently utilized enzymes and other biological molecules used within *in vitro* digestion models are pepsin, pancreatin, trypsin, chymotrypsin, peptidase, a-amylase, lipase, bile salt and mucin. Several studies have utilized enzymes collected from human subjects, whereas others have used enzymes extracted from animal or plant sources. For instance, Almaas *et al.* (2006) studied gastric juice and duodenal juice collected from human subjects. Chattertona *et al.* (2004) utilized gastric juice collected from human infants. It should be noted that most enzymes utilized for *in vitro* digestion studies are collected or extracted from omnivorous animals, i.e., pigs, rats, or human volunteers. The types of enzyme included within an *in vitro* digestion model tend to reflect the major food components being investigated, e.g., lipases for lipid digestion, proteases for protein digestion and amylases for starch digestion. Finally, it should be noted that different enzymes are usually added sequentially, rather than all together, so as to simulate the different steps of the digestive process. The activity of an enzyme preparation may decrease over time and so it is important to prepare them freshly for each study (Boisen & Eggum, 1991).

2.4.2 Digestion and transit time

The digestion time for each step (e.g., mouth, stomach and small intestine) is an important factor to establish when designing an appropriate*in vitro* digestion model.In vivo, the digestion time depends upon individual characteristics (age, sex, health status, mental state, time of day) and food properties (total amount, composition, particle size) and may vary quite considerably (McClements *et al.*, 2009). A short transit time of a food within the small intestine may limit the absorption of bioactive lipophilic compounds, thereby reducing their bioavailability (Dahan & Hoffman, 2008). Van Citers and Lin (1999) reported that lipids in the gastrointestinal tract delay the gastric emptying i.e., the gastric transit time is increased. Therefore, in the case of testing high-lipid food samples, enzymes (lipase or pancreatin) and bile salt/ phospholipid amounts and digestion time should be increased in an *in vitro* digestion system. *In vitro* digestion models do not usually take the large intestine into account, because the absorption of compounds mainly takes place mainly in the small intestine. Therefore, Brandon *et al.* (2006) reported that only the bioaccessibility determined in the chyme of the small intestine is relevant for risk assessment.

2.4.3 Effect of *in vitro* digestion on antioxidant properties of fruit extracts:

The health effects of polyphenols depend on the amount consumed and on their bioavailability. It is necessary to revise ideas in many *in vitro* experiments the components of phytochemical are best stable and can be transformed or changed into active metabolites by many factors from the gastrointestinal tract, e.g. environmental pH, kind of sugar present in the molecule and hydrophobicity. Under the alkaline conditions in the small intestine and digestion by pancreatin and bile, some of the flavanones from orange juice are transformed to insoluble chalcones (Izquierdo *et al.*, 2001). A study using a cultured cell model for quercetin glycosides absorption in the small intestine by Murota and Terao (2003) explained that the hydrolysis of the glucosides accelerated their absorption in the small intestine. The effect of digestive secretions on the stability of (+)-catechin, (-)-epicatechin and B2 and B3 dimers from a procyanidin-rich grape seed extract have been reported by Laurent *et al.*(2007), the availability of phenolic compounds was not affected by salivary and gastric digestions but decreased during intestinal digestion. Tarko *et al.* (2009) found that the polyphenolic compounds of selected fruits after digestion, polyphenols were hydrolyzed, especially glycosides of

quercetin and cyanidin. Phenolic acids and cyanidin were characterized by low availability for absorption, whereas catechin and quercetin had a very high level of accessibility in the model small intestine. Beard and Ryan (2010) showed that total antioxidant capacity of commercial tomato juices increased significantly ($p<0.05$) after both gastric and duodenal phase as measure by FRAP assay. Nevertheless, the results of the DPPH assay showed a small increase in total antioxidant capacity after the gastric phase and a small decrease after the duodenal phase. The beetroot juice shot had an increase total antioxidant capacity and total polyphenol content after *in vitro* digestion procedure with simulated gastric and duodenal phase (FRAP value and total polyphenol content increased after gastric digestion and remained higher following duodenal digestion) (Peter *et al.*, 2011). Courraud *et al.* (2013) evaluated two parameters during *in vitro* digestion of carotenoids and retinoids from carrot juice, raw and cooked spinach. The result showed that carrot juice provided amount of bioaccessible provitamin A versus in cooked and raw spinach. Moreover, Toydemir *et al.* (2013) found that anthocyanin bioavailability in the commercial cherry nectar (fruit drinks containing 25-99% fruit juice) production process was much higher than in the fresh fruit. Helal *et al.* (2014) investigated the in vitro bioaccessibility of polyphenols and formulations during simulated gastropancreatic digestion. The results showed the decline in total polyphenols was caused by the precipitation of tannins by pepsin. The addition of sweeteners (sucrose and honey) increased the polyphenols bioaccessibility decreasing the interaction bet ween pepsin and tannins. Moreover, milk addition proteolysis increases the Antioxidant capacity. The effect of thermal processing on the tomato and red carrot carotenoids transfer during digestion was studied. The effect of thermal on these systems depended on carotenoid species, being negative for lycopene but positive forβ-carotene (Palmero *et al.*, 2014). Furthermore, Stanisavljevic *et al.* (2015) founded that after *in vitro* digestion of chokeberry juice, although a large proportion of chokeberry phenolics undergo transformation during digestion they are still potent as antioxidant and antiproliferative agents.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental materials

Five underutilized fruits of Bangladesh *viz.* Amla (*Emblica officinalis*), Hog plum (*Spondias manigfera*), Elephant apple (*Dillenia indica*), Bilimbi (*Averrhoa bilimbi*) and Olive (*Elaeocarpus floribundus*) were selected in the present study to evaluate the bio-chemical attributes as well as antioxidant capacity. All the fruits were collected at harvest maturity from the well managed fruit's orchard of the Department of Horticulture, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. All the fruits were collected on the day of bio-chemical analysis.

Figure 3.1: Five indigenous raw fruits selected for the study (A = Amla, B = Hog plum, C = Elephant apple, D = Olive and E = Bilimbi)

3.2 Research location

The present research work was performed in the Food and Process Engineering Laboratory-1 and the Laboratory of Horticulture, HSTU, Dinajpur, Bangladesh.

3.3 Preparation of the Extracts

Five fruits of each species were selected for extraction. After harvesting, all the fruits were washed two times with tap water. Afterward, extraction was performed according to Swain and Hillis (1959) method with little modification. The same extraction method was followed for evaluating flavonol content, flavonoid content and antioxidant capacity.

Twenty five ml of methanol (Purity 99.8%, Merck, Damstadt, Germany) was added to three grams of each fresh fruit tissue separately and homogenized using homogenizer (VELP Scientifica, Italy). Then, the samples were kept at 4°C for 12 h followed by a centrifugation at 4000 rpm for 30 minutes using a centrifuge. The supernatants were collected and used for further analysis.

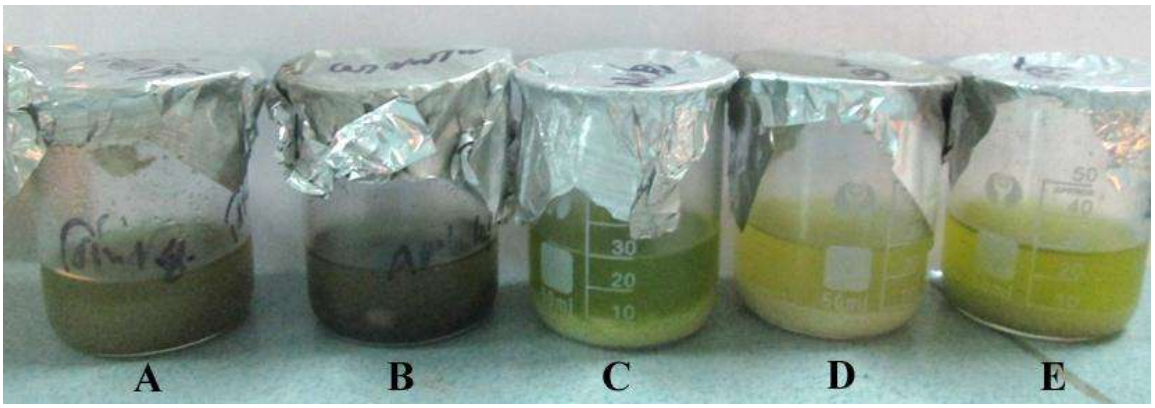

Figure 3.2: Five indigenous fruit's fresh extracts. (A = Bilimbi, B = Amla, C = Olive, D = Elephant apple and E = Hog plum)

3.4 Bio-chemical analysis of selected fruits

3.4.1 Determination of titrable acidity

Determination of titrable acidity was done according to the method described by Ruck (1969). At first, 1 gm of fresh fruit tissue was mixed with 10 ml of distilled water, slightly mashed and filtered. Then the filtrate was titrated against 0.1 N NaOH solution using phenolphthalein (2-3 drops) indicator until pink color was developed. The titrable acidity percentage was calculated by the following equation:

$$\% \text{ titrable acidity} = \frac{\text{Titre value} \times \text{normality of NaOH} \times \text{acid factor}}{\text{Weight of sample (gm)}} \times 100$$

3.4.2 Determination of pulp pH

The pH value of the selected fruits pulp was measured according to the method AOAC (2000). A digital pH meter (HANNA pH 211 Microprocessor pH meter, Romania) was used to determine the pH value of the sample by performing two point calibrations (with buffer 7.0 and buffer 4.0) before measuring the sample pH.

3.4.3 Determination of total soluble sugar, reducing sugar and non-reducing sugar

Preparation of fruit extracts

Fruit extract for total soluble sugar and reducing sugar analysis was prepared according to the method described by Saadati *et al.* (2013) with few modifications. At first, 500 mg fresh fruit pulp was taken in a 50 ml beaker. Afterward, 10 ml of 80% ethanol solution was added to it and homogenized. Then, the solution was centrifuged at 2000 rpm for 20 minutes. The extraction solution was prepared freshly on each analysis day.

Preparation of DNS (3,5–di-nitro salicylic acid) solution

One gram of NaOH was taken in a 100 ml beaker and 70 ml deionized water was added to it. Afterward, 18.2 g sodium-potassium tartarate (Merck, Mumbai, India) was dissolved in the solution. After dissolving, 1 g of 3,5–di-nitro salicylic acid (DNS) was added with it and stirred continuously. Then, 0.05 g Na_2SO_3 and 0.2 g phenol were dissolved with the solution, respectively.

3.4.3.1 Determination of total soluble sugar content (mg/100ml)

The total soluble sugar content of the selected fruits was determined by using the method of Dubois (1956). For this, at first 2 ml of supernatant was mixed with 1 ml of 5% phenol solution. Subsequently, 5 ml of 95.5% sulphuric acid was added to the samples. The test-tubes were then allowed to stand for 10 minutes and vortexed for 30 seconds. The test tubes were kept in water bath at room temperature for 20 minutes for colour development. Finally, the absorbance was recorded using a UV/VIS spectrophotometer (PG instruments T60, UK) at a wavelength 490 nm. Standard curve for the total soluble sugar determination was constructed by using glucose solutions whose concentrations ranged between 0 to 0.25 mg/ml.

Figure 3.3: Glucose solution with different concentrations (0-0.25) used for the development of standard curve for total soluble sugar determination.

3.4.3.2 Determination of reducing sugar content (mg/100ml)

Content of reducing sugar in the tissue of selected five fruits were analyzed by following the method suggested by Miller (1959). In a test tube, 0.5 ml fruit extract was mixed with 0.5 ml DNS solution. The mixture was then boiled for 10 minutes and cooled by immersing the sample containing test tube in cold water. Five ml of de-ionized water was then added to the test tube and mixed well. Afterward, the absorbance was taken at 540 nm in a UV-VIS spectrophotometer (PG instruments T60, UK). The standard curve was developed by using glucose solutions whose concentrations ranged between 0 to 1.2 mg/ml.

3.4.3.3 Determination of non-reducing sugar content (mg/100ml)

Non-reducing sugar content of the fruits was determined by subtracting the reducing sugar content from the total soluble sugar content by using the following formula:

Non- reducing sugar (mg/100 ml) = Total soluble sugar – Reducing sugar content

3.5 Determination of bioactive compound content of selected fruits

3.5.1 Analysis of ascorbic acid (mg/100g FW)

In the present study, the ascorbic acid content was determined using the spectrophotometric procedure (Bajaj and Kaur, 1941). Five grams of fruit tissue were homogenized with a homogenizer (VELP Scientifica, Italy) in 100 ml oxalic acid-EDTA (Ethylene di amine tetra acetic acid) cold solution. The homogenate was then centrifuged at 3000 rpm for 10 minutes and the supernatant was subsequently filtered with filter paper (Whatman No. 1). A 5 ml aliquot was then transferred to a 25-ml volumetric flask to which 0.5 ml metaphosphoric acid (Merck, Germany)-acetic acid solution, 1 ml sulphuric acid solution (5%) and 2 ml of ammonium molybdate (Merck, Germany) (5%) reagent were added. The mixture was adjusted to a volume of 25 ml with distilled water and allowed to stand for 15 minutes. After that the absorbance at 760 nm was measured with a UV-VIS spectrophotometer (PG Instrument Ltd. Model T60, UK). The ascorbic acid concentration was quantified using a standard curve of L-ascorbic acid and expressed as mg /100 g of fresh weight (FW).

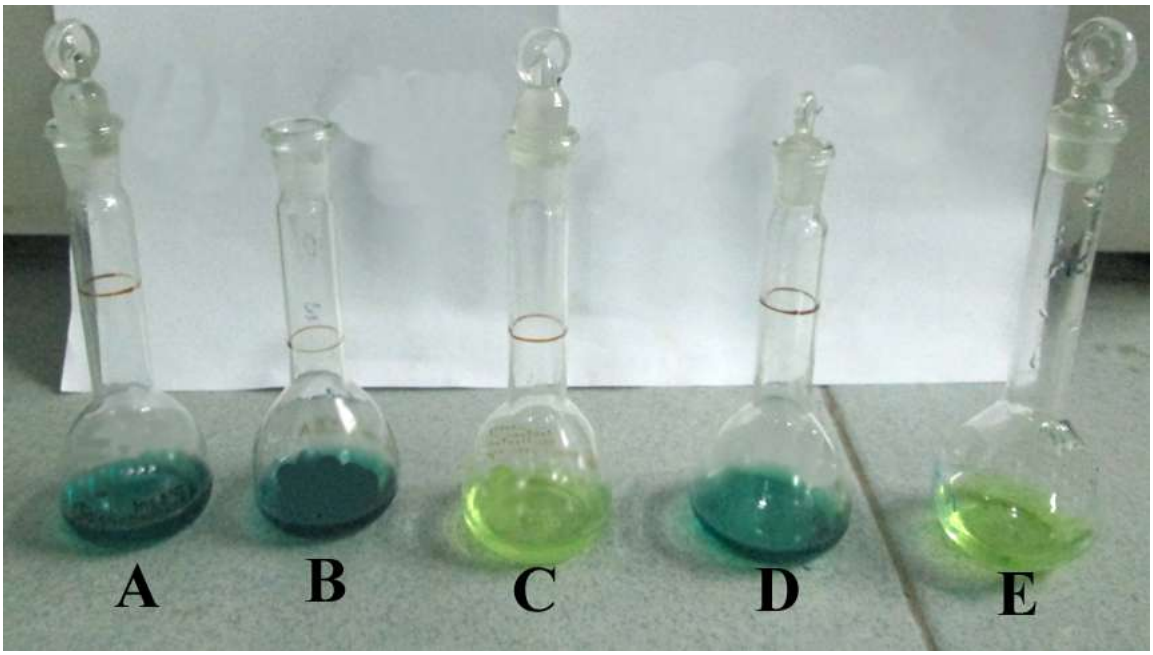

Figure 3.4: Estimation of ascorbic acid content. (A = Elephant apple, B = Amla, C = Olive, D = Bilimbi and E = Hog plum)

3.5.2 Determination of total polyphenol content (mg GAE/100 g FW)

Total phenol compound was quantified using Folin-Ciocalteu (FC) and the colorimetric method of Singleton and Rossi (1965). The extraction was done according to Velioglu *et al.* (1998) using 1 g fruit flesh. The fruit tissues were disrupted into the extraction medium using a homogenizer. The flesh was extracted with 4 ml 80% aqueous methanol containing 2.7% HCl (37%), shaken for 2 hours on an orbital shaker at 200 rpm in the room temperature and centrifuged at 5300 rpm for 15 minutes. Three hundred microliter (300 µl) of the extract was added to 2.25 ml of Folin–Ciocalteu reagent (Merck, Germany) followed by 2.25 ml of sodium carbonate solution (60g/l).

The samples were vortexed and left to stand for 90 min at the room temperature. After incubation, the absorbance was measured at 765 nm using a UV-VIS spectrophotometer (PG Instrument Ltd. Model T60, UK). The phenol content was then estimated using the standard curve of gallic acid (Merck, Germany) and the results were expressed as the mg of gallic acid equivalents (GAE) /100 g FW.

3.5.3 Estimation of total flavonol content (µg of quercetin equivalent /100 g FW)

Total flavonol content in the tissue of selected fresh fruit as well as the digested solution was determined according to the procedure described by Karunakaran and Kumaran (2007). Two ml of the sample extract was added to 2 ml of $AlCl_3$ (Merck, Germany) prepared in ethanol (2%). Afterward, 3 ml of (50 g/l) sodium acetate solution was added in it. Then the mixture was allowed to incubate for 2.5 h at 20°C. Finally, the absorbance was measured at 440 nm in a UV-VIS spectrophotometer (PG instruments T60, UK). Total flavonol content was calculated as µg of quercetin equivalent/100 g FW. All the samples were analyzed in triplicate.

3.5.4 Determination of total flavonoid content (mg of quercetin equivalent /100 g FW)

Aluminum chloride based colorimetric assay (Liu *et al.*, 2008) was used to determine the total flavonoid content in the tissue of selected fruit and *in-vitro* digested solution, too. An aliquot of 2 ml was mixed with 0.2 ml of 0.5% sodium nitrate and incubated for 5 min. Then, 0.2 ml of 10% aluminum chloride (Merck, Germany) was added to the mixture and mixed well. After 6 minutes, 2 ml of 1M sodium hydroxide was added to the mixture. The mixture was made up to 5 ml with 80% methanol and stirred thoroughly.

The absorbance of the mixture was recorded at 510 nm using a UV-VIS spectrophotometer (PG instruments T60, UK). Quercetin (0–0.2 mg/ml) was used to plot a calibration curve for quantification. The results of total flavonoids content were expressed as mg quercetin equivalents (QE)/100 g FW (Chew *et al.*, 2011).

3.6 Determination of antioxidant capacity

3.6.1 ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)) assay

The $ABTS^+$ assay was carried out according to the procedure of Re *et al.* (1999). The assay is based on the ability of an antioxidant compound to quench the $ABTS^+$ relative to that of reference antioxidant i.e. Trolox. A stock solution of $ABTS^+$ was prepared by mixing ABTS (Sigma-Aldrich, Steinheim, Germany) solution with a potassium persulfate (Merck, India) solution at 7 mM and 2.45 mM final concentrations, respectively. The mixture was then maintained in dark room at room temperature for 16 hours before use. The fresh $ABTS^+$ working solution was prepared by dilution of 5 mM PBS (pH 7.4) of the stock solution to achieve an absorbance of 0.40 at 734nm. Trolox solution (1.5 mM) was prepared by dilution of PBS.

An aliquot of 100µl of diluted extract was added to 1900 µl of $ABTS^+$ working solution. For the blank and standard curve, 100µl of Trolox solution were used in exchange of sample. Absorbance was measured by means of a UV-VIS spectrophotometer (PG Instrument Ltd. Model T60, UK) at 734nm immediately after addition and rapid mixing.

Figure 3.5: ABTS solution

3.6.2 Ferric-reducing antioxidant power (FRAP) assay

According to the method of Hemalatha and Kumar (2011), the reducing power of the selected fruit's extract was evaluated with slight modifications. The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide [$(K_3Fe(CN)_6$] (Qualikems, India) (1% w/v) was added to 1 ml of each of the extracts. The resulting mixture was incubated at 50°C for 30 min, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the supernatant of the solution. A volume of 2.5 ml supernatant solution was mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (Merck, Germany) (0.1%, w/v). The absorbance was then measured at 700 nm with a UV-VIS spectrophotometer against blank sample. Standard curve was constructed using $FeSO_4.7H_2O$ in the concentrations ranged between (0.1-1.0) mM. Increased absorbance of the reaction mixture indicated higher reducing power of the fruit extract.

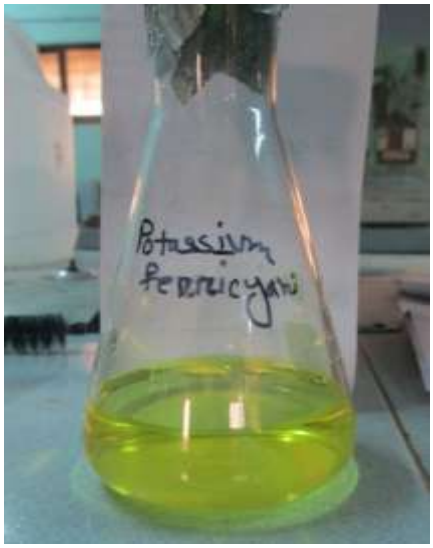

Figure 3.6: Potassium ferricyanide solution

3.6.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH free radical scavenging activity of the methanol extracts of the selected fruits were analyzed according to the method suggested by Sanchez-Moreno *et al.* (1998). An aliquot of the fruit extract (0.1 ml) was added to 3.9 ml of DPPH (Sigma-Aldrich, Steinheim, Germany) solution containing 0.025 g/L in methanol. Samples absorbance was measured at 515 nm after 30 minutes of incubation in darkness. DPPH solution violet color disappeared in the presence of antioxidant, as a result of free radical

scavenging in measured medium. The standard curve for this assay was also constructed using Trolox (Sigma-Aldrich, Steinheim, Germany) in the concentrations ranging from 0 to 0.330 mM. The dilution of the trolox solution was made by using PBS (phosphate buffer saline) solution whose pH value was 7.4.

Figure 3.7: DPPH solution

3.7 Gastrointestinal *in vitro* digestion

Crude extracts of 5 selected fruits were prepared in triplicate followed by digestion *in vitro* based on the methodology described by Faller *et al.* (2012), with a few modifications. Two ml of crude fruit extract was mixed in a saline solution containing 140 mM NaCl, 5 mM KCl (Merck, India) and 150 mM BHT (Merck, Germany) at a ratio of 1:4 v/v (sample/saline) to obtain a final volume of 14 ml. This was followed by agitation at 21°C for 10 min. Afterward, the mixture was acidified to pH 2.0 with 0.1 M/1 M HCl and was added to a 0.25 ml solution containing pepsin (Loba Scientific, India) (0.2 g in 5 ml 0.1 M HCl) and the samples were incubated at 37°C with stirring for 1 h in a shaking incubator (Vision Scientific, model: VS-8480SN, South Korea).

After step-wise gastric digestion, the intestinal digestion of the sample was commenced by raising the pH to 6.9 by the addition of 1 M $NaHCO_3$/0.1 M $NaHCO_3$. Then, 1.25 ml of bile (Loba Scientific, India) and pancreatic solution (containing 0.225 g bile extract and 0.0375 g pancreatin (Merck, Germany) in a volume of 18.75 ml 0.1 M $NaHCO_3$) was added followed by the incubation with shaking at 37^{o}C for 2 h. The final volume of

the digested sample was adjusted to 14 ml by the addition of brine. The samples were filtered and analyzed again by the methods described in the sections 3.5.3, 3.5.4, 3.6.1, 3.6.2 and 3.6.3.

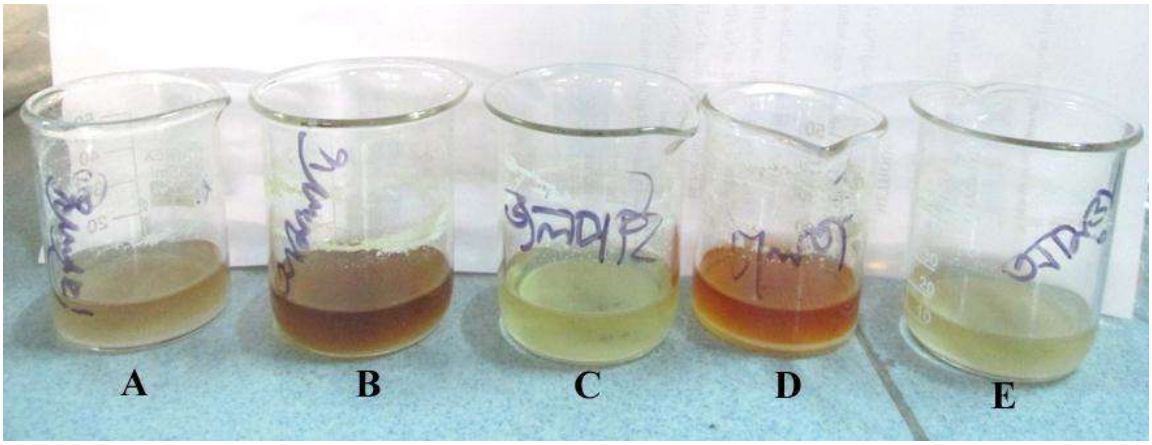

Figure 3.8: *In vitro* digested extracts of selected five fruits. (A = Bilimbi, B = Amla, C = Olive, D = Elephant apple and E = Hog plum)

3.8 Statistical analysis

For each experiment (both before and after *in vitro* digestion), one factor analysis of variance (ANOVA) was conducted for all variables using the statistical software SPSS (version 22). All analysis were carried in 3 replicates. The results were presented as mean ± SE. The means were compared using Fisher's Least Significant Difference (Lsd). All analyses were regarded as significant at $p \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Findings of the current research in terms of biochemical properties and bioactive compound along with antioxidant capacity of selected fruits discussed below:

4.1 Bio-chemical properties

The important bio-chemical properties of the selected five indigenous fruits are summarized in Table 4.1. These parameters are discussed separately in below:

4.1.1 Pulp pH

Regarding pH value of the pulp of selected fruits, a significant difference ($p<0.05$) was found which ranged from 2.79 to 3.96 (Table- 4.1). Highest and lowest value of pulp pH was found in Bilimbi (3.96) and Amla (2.79), respectively. However, the similar result was also reported by Shantaram (2013) who found lower pH (2.16) value in a local cultivar of Bilimbi in India. The present result is in accordance with the findings of Parveen and Khatkar (2015) who found pH value 2.82 and 2.84 in 'Kanchan' and 'Banarasi' Amla variety, respectively.

4.1.2 Titrable acidity

Table 4.1 shows that the titrable acidity of the selected fruits varied significantly ($p<0.05$) from 0.34 to 1.81%. The highest value of titrable acidity 1.81% was found from Amla and lowest value from Bilimbi (0.34%). In case of Amla, Singh *et al.* (2016) recorded lower titrable acidity than the present findings whereas, Bakshi (2015) found higher titrable acidity. Compare to the present findings, this contradiction might be due to the use of different cultivars of Amla.

4.1.3 Total soluble sugar (mg/100ml)

A significant difference ($p < 0.05$) in case of total soluble sugar was noted among the samples (Table 4.1). Considering total soluble sugar, Amla (32.67 mg/100ml) differed from the others by presenting the highest total soluble sugar, followed by Hog plum, Elephant apple, Olive and Bilimbi. This result is in line with the opinion of Parveen and Khatkar (2015) who found almost similar total sugar (32.85 mg/100ml) for NA-7 Amla

Table 4.1: Biochemical properties of selected five fruits

Sample	Parameter				
	pH	Titrable acidity (%)	Total sugar (%)	Reducing Sugar (%)	Non Reducing Sugar (%)
Amla	2.79 ±.006 [e]	1.81 ± .023 [a]	32.67 ±.0015[a]	26.33 ±.003[b]	6.34±0.81[c]
Elephant apple	3.88 ± .006[b]	0.36 ±.020 [d]	29.07 ±.0003[c]	27.00 ±.010[ab]	2.07 ±0.19[e]
Hog plum	3.20 ±.012 [c]	0.49 ± .020 [c]	30.00 ±.0006[b]	27.33 ±.003[a]	2.67 ±0.97[d]
Olive	2.85 ±.012 [d]	0.75 ±.023 [b]	28.83 ±.0007[d]	19.33 ±.002[c]	9.50 ±0.65[b]
Bilimbi	3.96 ±.006 [a]	0.34 ±.020 [d]	25.43 ±.0003[e]	7.00 ±.050[e]	18.43 ±1.78[a]
L.s.d	0.036	0.036	0.001	0.001	0.002

Results are expressed as mean ± SE.

[a–e] Different superscript alphabets in each column indicate significant difference among the fruit samples ($p < 0.05$).

variety. Previous study by Nayak *et al.* (2012) reported lower concentration of sugar (6.8 mg/100ml) for same Amla variety.

However, lowest value (25.43 mg/100ml) of total soluble sugar recorded in Bilimbi Comparing this result with literature, little lower amount (15.26 mg/100ml) was found by Laulloo *et al.* (2003) in case of green grape.

4.1.4 Reducing Sugar (mg/100ml)

It is evident from the data presented in Table 4.1 that reducing sugar content varied from 7.00 to 27.33 mg/100ml and showed a significant ($p<0.05$) difference among the samples. Among the five underutilized fruits studied, Hogplum (27.33 mg/100ml) showed to have highest value. The result is comparable with the findings of Akther *et al.* (2012) who observed lower reducing sugar (5.02 mg/100ml) for Barishal Hog plum. Variation in concentration of reducing sugar might be due to different cultivars as well as different agro-climatic conditions. Akther *et al.* (2012) reported variation in physico - chemical composition between Barishal and Mymensingh Hog-plum which might be due to the variation of soil, growing condition, harvesting period, maturity stage, climate etc.

Regarding the reducing sugar, the lowest value was observed in Bilimbi (7 mg/100ml). This result is little higher than the finding of Bakshi *et al.* (2015) who observed 3.41 mg/100ml reducing sugar in 'Neelam' cultivar of Amla.

4.1.5 Non -Reducing Sugar (mg/100ml)

According to the results presented in Table 4.1, mean value of non-reducing sugar ranged from 2.07 to 18.43 mg/100ml. The data shows that there is a significant ($p<0.05$) difference among the fruit samples. Maximum non-reducing sugar 18.43 mg/100ml was recorded in Bilimbi. These results were in accordance with Parveen and Khatkar (2015) who accounted closer result (15.26 mg/100ml) for 'NA-7' Amla variety. Elephant Apple (2.07 mg/100ml) obtained the lowest value in terms of non-reducing sugar. The similar findings were also described by Mahapatra *et al.* (2012) who reported little lower (1.45 mg/100ml) value for papaya. Variation in non-reducing sugar content might be due to different agro-climatic condition. These findings are in agreement with the findings of Akther *et al.* (2012).

4.2 Bioactive Compound Content

4.2.1 Total polyphenol (mg of GAE/100 g FW)

The amount of total polyphenol (TP) was found to be significantly ($P<0.05$) diversed among the fruit samples (Table 4.2). The phenolic content of the selected fruits ranged from 346.28 to 379.36 mg of GAE/100 g FW. The results presented in Table-4.2, indicated that Elephant apple (379.36 mg of GAE/100 g FW) contained highest polyphenols among the sample and olive scored the lowest (346.28 mg of GAE/100 g FW) one. The finding obtained in case of elephant apple is consistent with Kriengsak *et al.*(2006) who described the nearly similar (344.97 mg of GAE/100 g of fresh weight) result for guava in terms of total polyphenol. In the present study, the phenolics concentration of Elephant apple evaluated higher than that found by other authors in other fruits: Hassimotto *et al.* (2005) reported the following data for phenolics (mg of GAE/100 g of fresh weight): pineapple (67.2±0.6), soursop (120.0±8.0) and murici (67.0±3.0). However, the minimum total phenolics was accounted for olive i.e, 346.28 mg of GAE/100 g FW which is higher than the result (299.35 mg of GAE/100 g FW) reported by Susamci *et al.* (2016) in 'Humra' (salt free) olive collected from Godence, Turkey . The variation in total phenolics content might be due to cultivation locations, seasonal variation, agricultural practices (Patumi *et al.*, 2002; Marsilio *et al.*, 2005; Vinha *et al.*, 2005). The phenolic compound content might also be affected by factors such as genotypes and different levels of maturity (Petridis and Therios, 2012).

Table 4.2: Expression of total phenol content and ascorbic acid content of five fruits

Sample	TPC (mg of GAE/100g FW)	Ascorbic acid (mg/100g FW)
Amla	351.57 ± 0.11^{cd}	523.31 ± 0.81^{a}
Elephant apple	379.36 ± 0.61^{a}	106.38 ± 0.19^{b}
Hog plum	370.79 ± 0.17^{ab}	60.72 ± 0.97^{c}
Olive	346.28 ± 0.54^{d}	45.62 ± 0.65^{e}
Bilimbi	363.70 ± 0.30^{bc}	51.59 ± 1.78^{d}
Lsd	8.29	2.04

Results are represented mean ± SE.

[a–e] Different superscript alphabets in each column indicate significant difference among the fruit samples ($p < 0.05$).

4.2.2 Ascorbic acid (mg/100g FW)

Vitamin C is a powerful water-soluble antioxidant. It is called an antioxidant because by donating electrons and prevents other compounds from being oxidized. The content of vitamin C among the five fruits differed significantly ($p \leq 0.05$) from 45.62 to 523.31 mg/ 100 g of FW (Table 4.2). The maximum amount of vitamin C was recorded in Amla (523.31 mg/ 100 g FW) and it was significantly higher than all other fruits. This finding was higher than the results stated by Singh *et al.* (2012) and Bakshi *et al.* (2015) who found 486 and 480.20 mg/100 g FW in 'Desi' variety of Amla, respectively. variation in ascorbic acid content might be due to the physicochemical changes that occur with the degree of maturation. During these changes, other organic acid may also be degraded with the increase in pH as well as lowering of acidity (Wills, 1981). Olive was found to have lowest ascorbic acid content (45.62 mg/100 g FW).

4.2.3 Flavonoid content (mg QE/100 g)

Flavonoids are found to possess strong antioxidant activities. It is widely distributed in fruits and vegetables and exerts multiple biological effects including free radical-scavenging activity. It might be through providing protection on the DNA by forming complex with chelating metal ions such as: copper or iron which finally prevents the formation of reactive oxygen species (Souza and Giovani, 2004; Armida *et al.*, 2005). Estimated values of the total flavonoid content (TFC) in the fruit tissues are given in Figure 4.1. As shown in Figure 4.1, a significant ($p<0.05$) difference among the samples was observed in both undigested and digested stage. Conforti *et al.* (2008) reported that variation in total flavonoid content among the plant extracts might be due to the presence of other constituents and/or the presence of different types of phenols.

Amla differed from the others by presenting the highest flavonoid content and in both undigested (51.12 $\pm$ .007) and digested (53.03$\pm$ .003 mg QE/100 g) condition. All fruit samples exhibited a significant ($P<0.05$) increase in the TFC after *in vitro* digestion (Figure 4.1). Similar result was also observed by Goh and Barlow (2004) in Ginkgo biloba leaves and by Pavan *et al.* (2014) in papaya extracts. However, a contradictory report also concluded by Mosele *et al.* (2015) who reported a decreasing trend in total flavonoid concentration in the duodenal digests of pomegranate co-products.

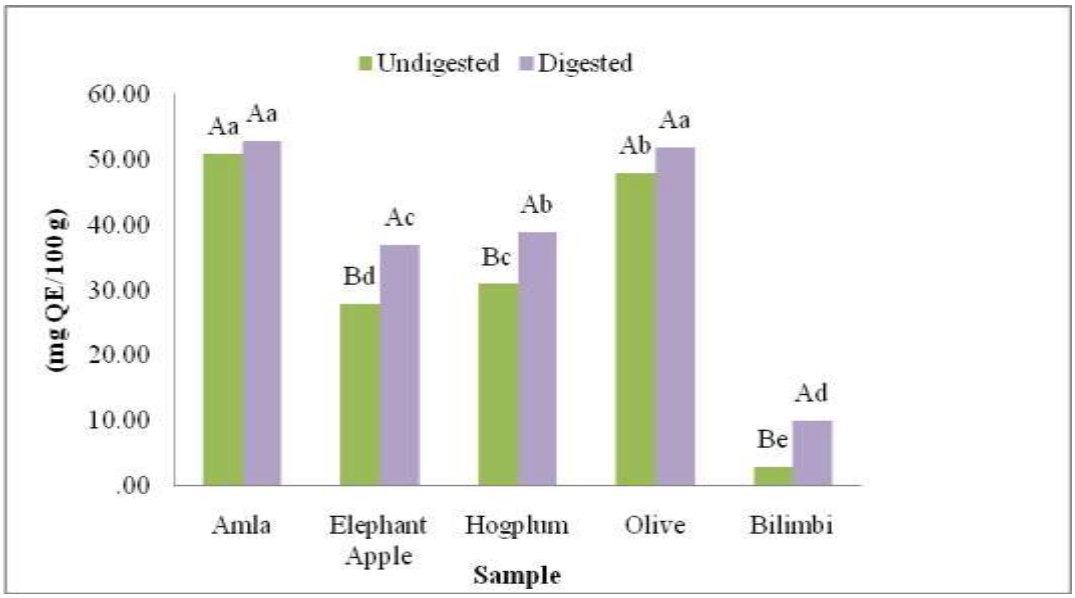

Figure 4.1: Effect of *in vitro* digestion on flavonoid content of selected fruits.

[a–e]Means followed by different alphabets are significantly different among samples ($p < 0.05$).

[A-B]Means followed by different alphabets are significantly different between the undigested and digested extracts of the same fruit ($p < 0.05$).

4.2.4 Flavonol content (µg QE/100 g)

According to the results presented in Figure 4.2, mean value of flavonol ranged from 42.99 to 480.82 before digestion and 40.00 to 200.05 µg QE/100 g after digestion. In respect of flavonol content, fresh undigested extract differed significantly ($p < 0.05$) among the five fruits. The highest value of flavonol obtained from Elephant apple and lowest from Bilimbi both in the undigested and digested extracts (Figure 4.2). The present findings are in accordance with Sultana and Anwar (2008) who stated little higher result (359.4 mg QE/100g) for Mulberry.

This study has demonstrated that flavonol content significantly ($p<0.05$) decreased during a simulated digestion except in Bilimbi. This decrease in flavonol content might be attributed to the explanation that some antioxidants could be rendered more reactive at acidic pH in the gastric phase of *in vitro* digestion (Beard *et al.*, 2011; Bermu´ dez-Soto *et al.*, 2007; McDougall *et al.*, 2005 a, b). It is possible when exposed to such conditions a proportion of the compounds are transformed into different structural forms with different chemical properties which might not be identified by the employed method (Ryan and Prescott, 2010).

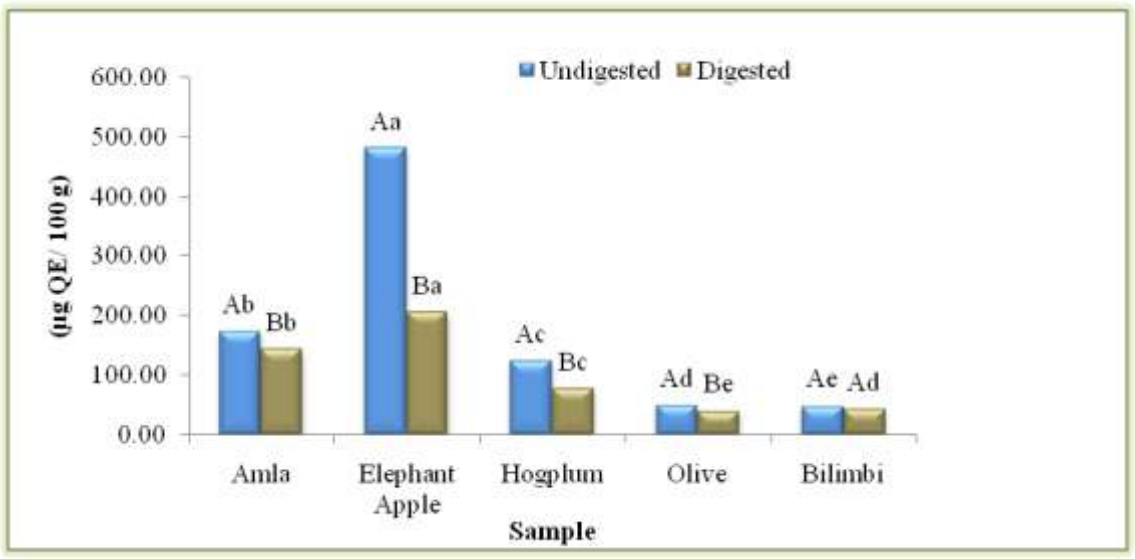

Figure 4.2: Effect of *in vitro* digestion on the flavonol content of selected fruits.

[a–e]Means followed by different alphabets are significantly different among samples ($p < 0.05$).

[A-B]Means followed by different alphabets are significantly different between the undigested and digested extracts of the same fruit ($p < 0.05$).

4.3 Antioxidant capacity study

4.3.1 DPPH (% inhibition)

Rearding DPPH radical scavenging assay, antioxidants react with DPPH$^{\bullet}$ by donating hydrogen and reduced it to the yellow colored DPPH from violet colored DPPH$^{\bullet}$. The degree of the discoloration indicates the radical scavenging potential of the sample (Blois, 1958). In case of undigested extracts the maximum inhibition (92.00 %) was recorded in elephant apple, followed by Olive > Amla > Hog plum > Bilimbi (Figure 4.3). Regarding undigested fruit extracts the results showed that Amla, Elephant apple and Olive exerted significantly ($p<0.05$) higher DPPH % inhibition than that of Bilimbi and Hog plum. However, difference among the fresh extracts of Amla, Elephant apple and Olive was statistically identical (Figure 4.3). DPPH % inhibition value of the present study was varied from (85.67 to 92.0)% and (10.0 to 46.67)% in undigested and digested fruit extracts, accordingly (Figure 4.3). Similar inhibition value was also observed in pomegranate juice (Ryan and Prescott, 2010).

Regarding the digested extract, a significantly ($p<0.05$) decreasing trend in DPPH$^{\bullet}$ scavenging activity was recorded in digested extracts (Figure 4.3). This could be presumably due to the dependency of phenolic activity on pH of the digestion medium as reported by Wootton-Beard *et al.* (2011). They concluded that, pH of a substance

affected racemization of molecules, probably creating two chiral enantiomers with different reactivity which consequenly rendered some antioxidants more reactive at acidic pH in the gastric phase and less reactive at alkaline pH during the duodenal phase of *in vitro* digestion. The similar decreasing trend in DPPH scavenging activity was also verified by Fawole and Opara (2016) in pomegranate co-products. But a contrary result was observed by Beard *et al.* (2011) who claimed increasing trend of DPPH scavenging activity in 23 vegetables.

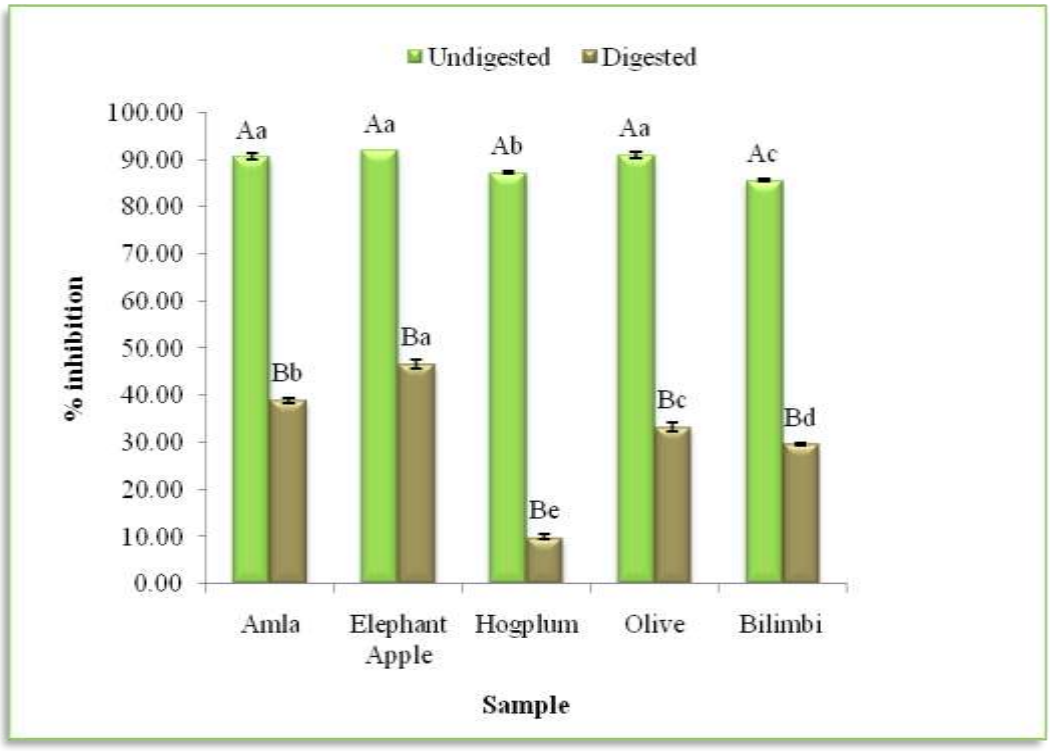

Figure 4.3: Effect of *in vitro* digestion on DPPH assay (% inhibition).

[a–e]Means followed by different alphabets are significantly different among samples ($p<0.05$).

[A-B]Means followed by different alphabets are significantly different between the undigested and digested extract of the same fruit ($p <0.05$).

4.3.2 $ABTS^{\bullet+}$ radical cation scavenging activity

In this assay, the capacity of selected extracts to scavenge the ABTS radical ($ABTS^{\bullet+}$) was assessed. This assay is based on the scavenging of the relatively stable blue ABTS radical ($ABTS^{\bullet+}$**)** converting it into a colourless product. The degree of decolourization reflects the amount of $ABTS^{\bullet+}$ that has been scavenged. According to the results, there was a significant ($P<0.05$) variation in the percentage inhibition of the undigested fruit extracts was observed**.** It was also observed that percentage inhibition was highest in

Elephant apple. On the other hand, Hog plum (88.67%) and Olive (88.42%) showed significantly lowest percentage inhibition than other three fruit species (Figure 4.4).

After *in vitro* digestion, the inhibition values of all fruit samples were decreased significantly ($p<0.05$). This could be attributed to the free radical reducing or scavenging capacity of bioaccessible phenolics present in the extracts during intestinal digestion as reported by Fawole and Opara (2016). Diminution in percent inhibition may also be attributed to the dependency of phenolic activity on pH of the digestion medium. Because, radical scavenging activity is mainly dependent on the number and position of hydrogen-donating hydroxyl groups on the aromatic rings of the phenolic compounds and it is believed that transition from acidic to alkaline environment enhances the Antioxidant capacity of phenolics through deprotonation of hydroxyl moieties present on their aromatic rings.(Tagliazucchi *et al.*, 2010 ; Bouayed *et al.*, 2011). The results are also consistence with Fawole and Opara (2016) who stated similar condition in case of vegetables. However, a contradictory result was also reported by Beard *et al.* (2011) for vegetable juices compare to our findings.

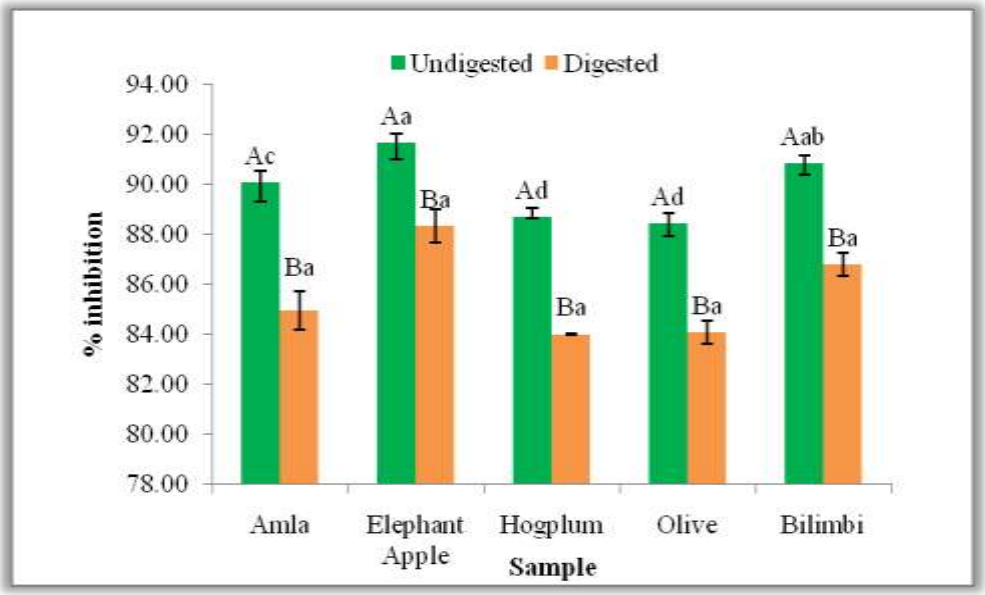

Figure 4.4: Effect of *in vitro* digestion on ABTS assay (% inhibition)

[a–d]Means followed by different alphabets are significantly different among samples ($p<0.05$).

[A-B]Means followed by different alphabets are significantly different between the undigested and digested extract of the same fruit ($p < 0.05$).

4.3.3 Ferric reducing Antioxidant power (FRAP)

FRAP assay is another important method of assessing antioxidant power. The initial FRAP values of the tested undigested fruit extracts varied significantly ($p< 0.05$) among the fruits within the range from 1.74 to 12.66 mg $FeSO_4$ .7 H_2O/ml (Figure 4.5). Amla exhibited the greatest FRAP value (12.66 mg $FeSO_4$ .7 H_2O/ml) to the other fruits. Elephant apple, Bilimbi, Olive and Hog plum had 4.39, 3.24, 2.88 and 1.74 mg $FeSO_4$ .7 H_2O/ml, respectively. After *in vitro* digestion, FRAP values for all fruit samples were significantly ($p< 0.05$) increased (Figure 4.5). After *in vitro* digestion the maximum value (14.68 mg $FeSO_4$ .7 H_2O/ml) was also exerted by amla and hog plum attained the minimum (2.19 mg $FeSO_4$ .7 H_2O/ml). The increasing trend of FRAP value after intestinal value of the present study was in accordance with the opinion given by Kumar *et al.* (2013) who found an elevation in the FRAP value in Pine apple and pomegranate juice. They also reported that the elevation might be as a result of other Antioxidant compounds except phenolics and flavonoids such as : organic acid and vitamins.

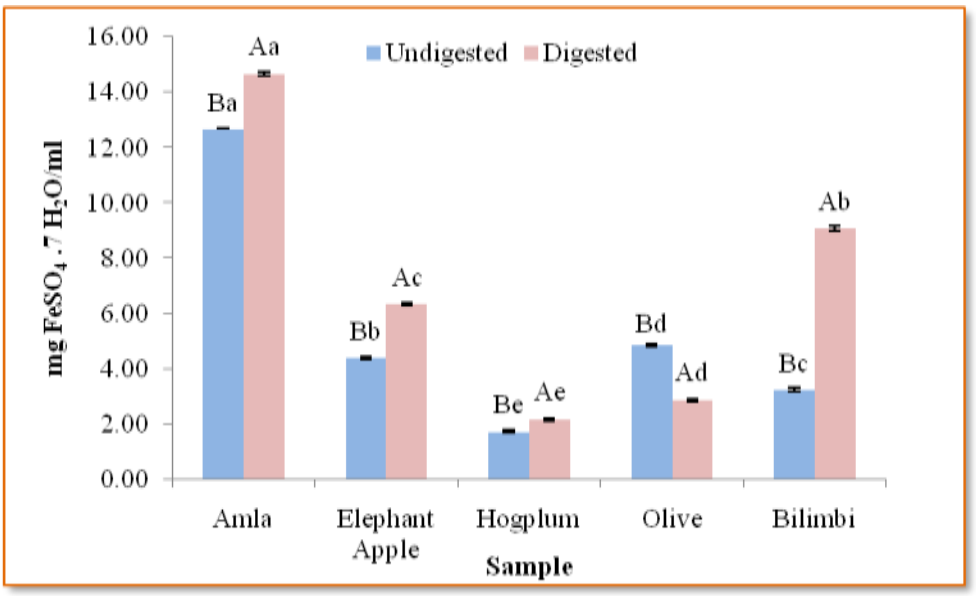

Figure 4.5: Effect of *in vitro* digestion on Ferric reducing Antioxidant Power

[a–e] Means followed by different alphabets are significantly different among samples ($p <0.05$).

[A-B]Means followed by different alphabets are significantly different between the undigested and digested extract of the same fruit ($p <0.05$).

CHAPTER V

SUMMARY AND CONCLUSION

Present study examined biochemical properties, bioactive compound and the effect of *in vitro* gastrointestinal digestion on the antioxidant capacity of five indigenous fruits grown in Bangladesh. A significant ($p < 0.05$) differences showed for all indices among the fruits. Amla attained maximum value of titrable acidity, total sugar and non-reducing sugar compared to other four fruits. Highest value of pH and reducing sugar was found in olive and hog plum, respectively.

Regarding the bioactive compound, Amla and Elephant apple exerted superior containment than that of Hog plum, Bilimbi and Olive in both undigested and digested extract. Highest (379.36 mg of GAE/100 g FW) total phenolics content was showed by Elephant apple whereas amla attained maximum (523.31 mg/100 FW) ascorbic acid content. Amla and Elephant apple contained highest amount of flavonoid and flavonol, respectively. In case of antioxidant capacity, maximum percent inhibition to DPPH and ABTS radical was shown by Elephant apple whereas Amla accounted uppermost value of ferric reducing antioxidant power (FRAP). Results of this study revealed that antioxidant capacity of all the fruits influenced significantly ($p<0.05$) by *in vitro* digestion.

The results of the current study to be concluded, the studied five fruits Amla and Elephant could be considered as enormous sources of antioxidants to prevent the degenerative effects of various chronic diseases caused by imbalanced free radical formation. However, this research recommends further investigation through in vivo feeding trials to reveal antioxidant capacity within the living organism.

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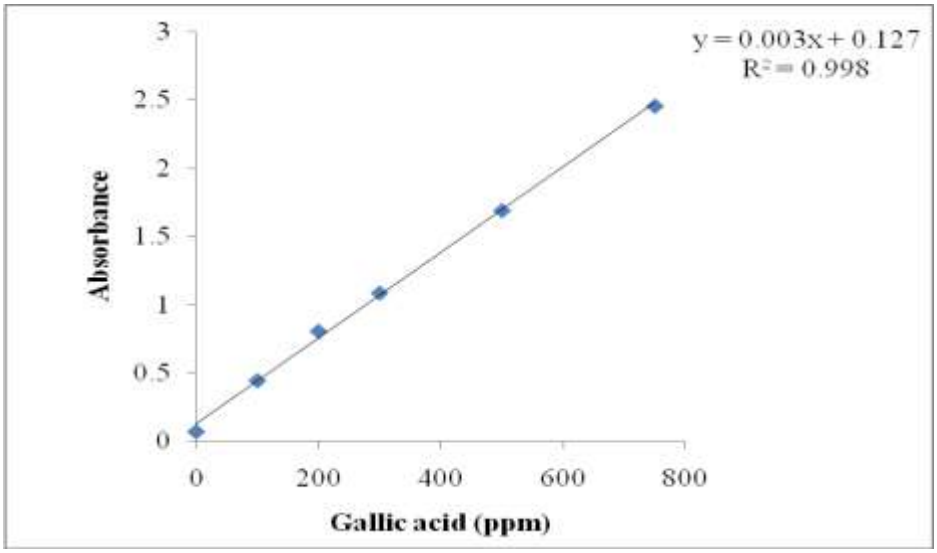
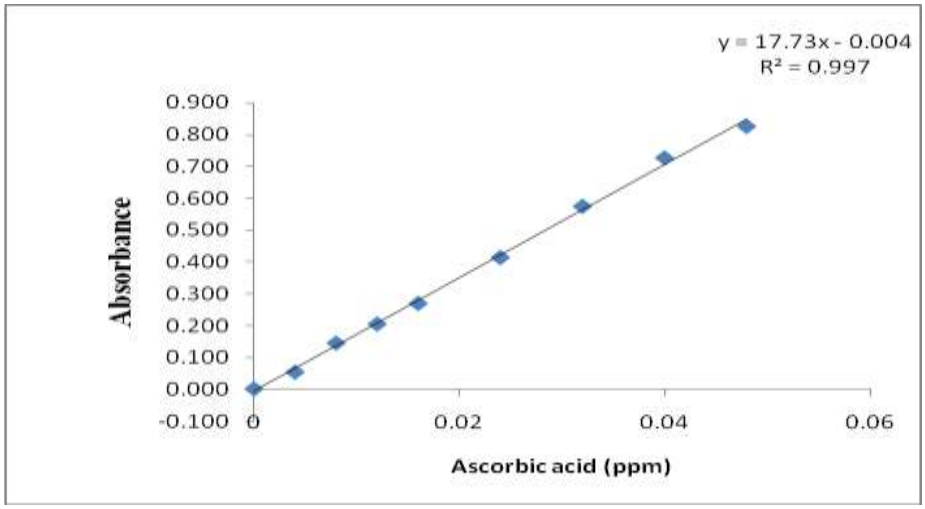
APPENDICES

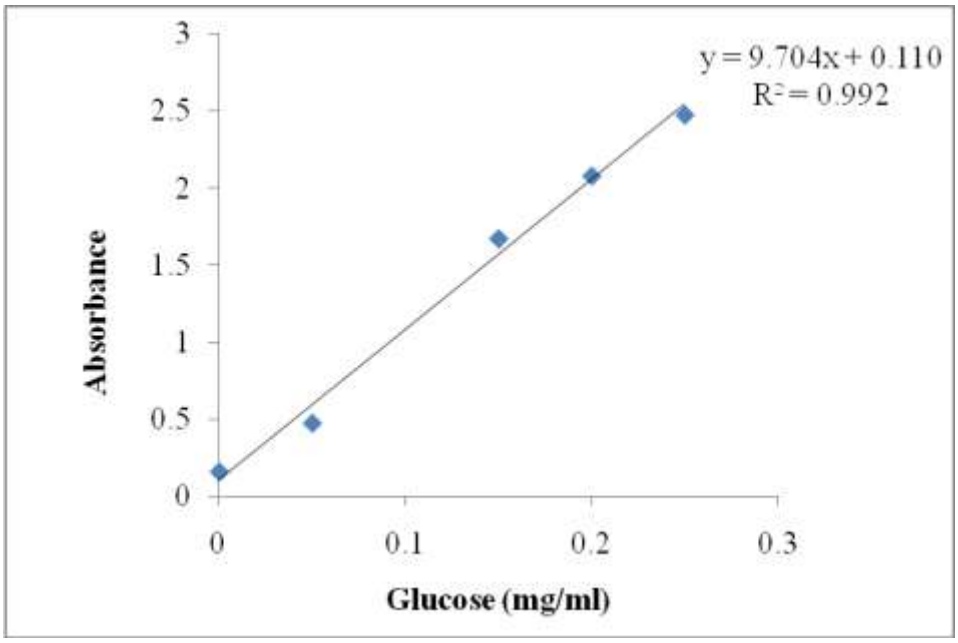
Appendix I : Standard Curve for Total Phenol



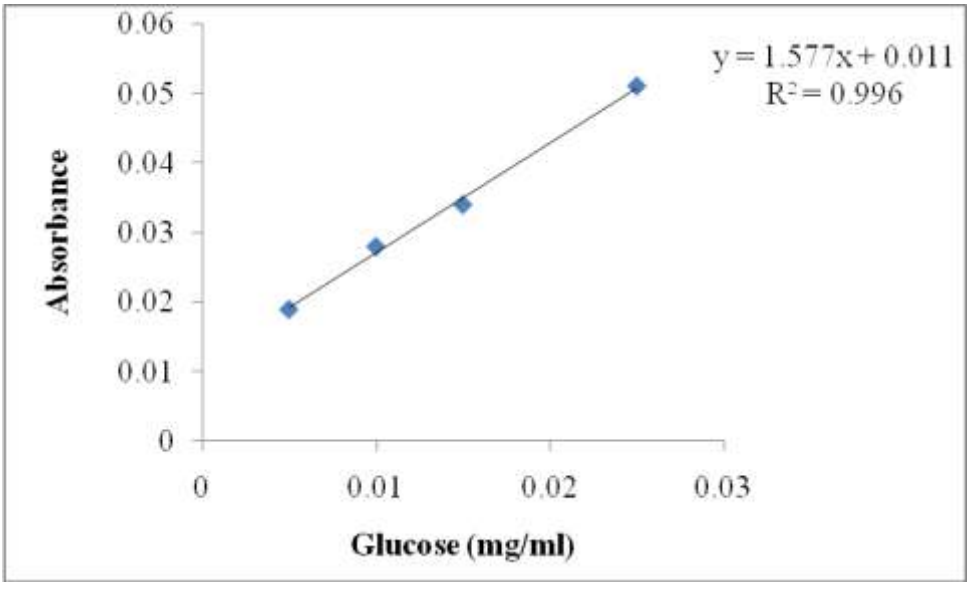
Appendix II : Standard Curve for Ascorbic Acid



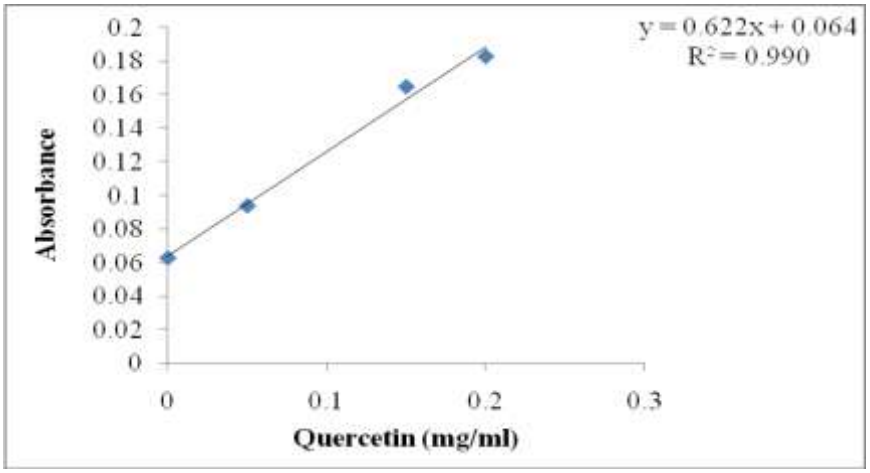
Appendix III : Standard Curve for Total soluble Sugar



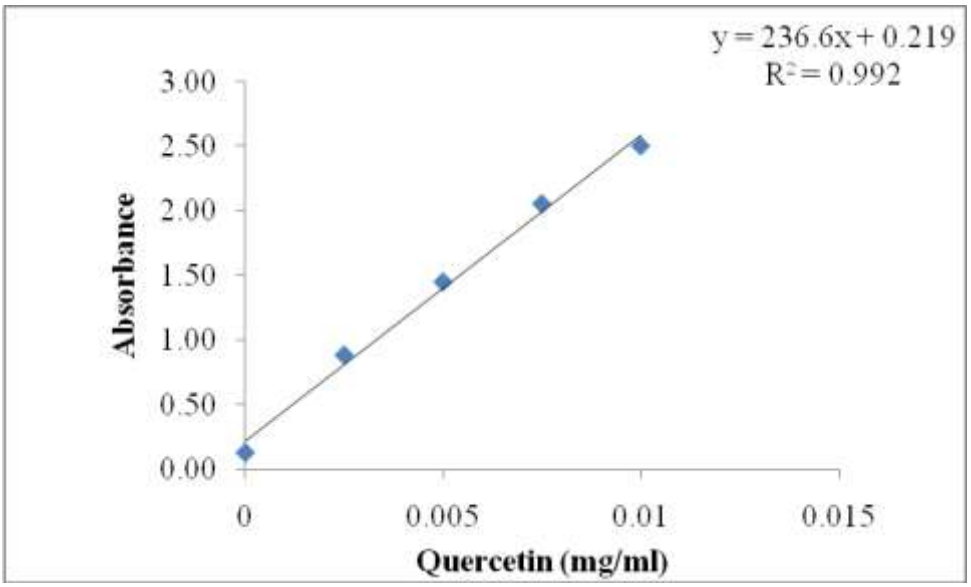
Appendix IV: Standard Curve for Reducing Sugar



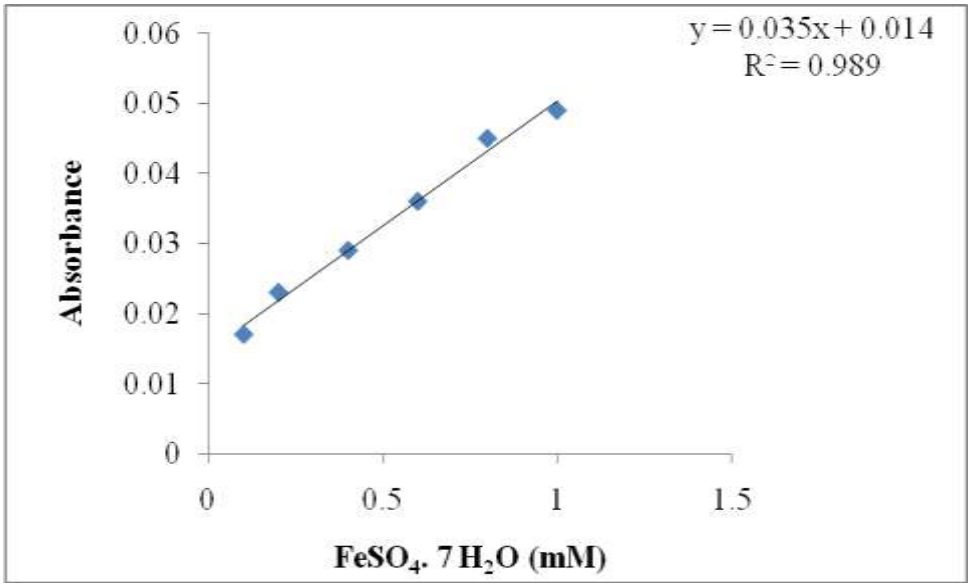
Appendix V : Standard Curve for Flavonoid



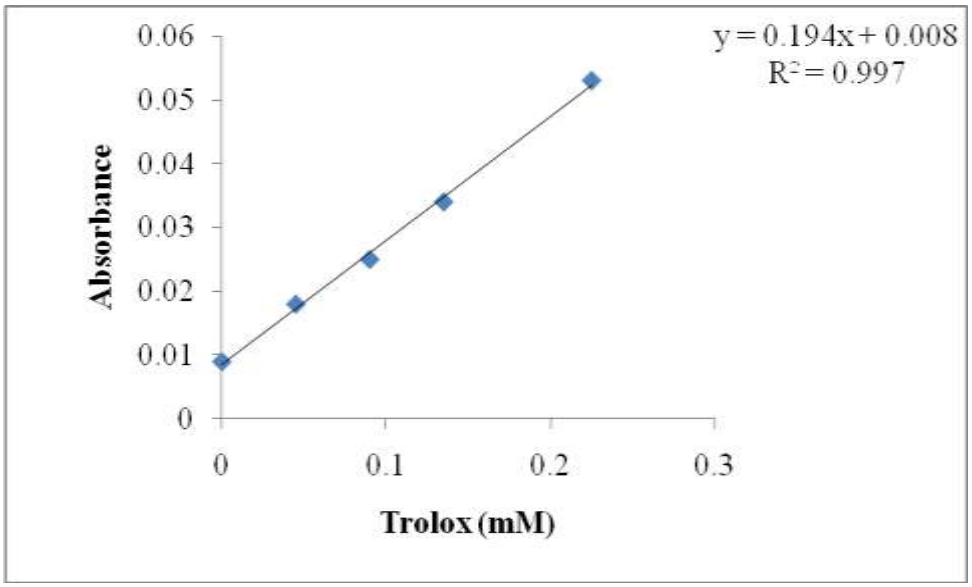
Appendix VI : Standard Curve for Flavonol



Appendix VII: Standard Curve for FRAP



Appendix VIII : Standard Curve for ABTS and DPPH



Appendix IX: Preparation of some important solutions:

a) Preparation of 140 mM NaCl solution

Molecular weight of NaCl = 58.44 g

1 liter 1 M solution required = 58.44 g NaCl

1 liter 140 mM solution required = 58.44 × 140/ 1000 = 8.1816 g NaCl.

So, 8.1816 g NaCl was dissolved in small amount of de-ionized water and volume was made 1 liter by water.

b) Preparation of 5 mM KCl solution

Molecular weight of KCl = 74.5513 g

1 liter 1 M solution required = 74.5513 g KCl

1 liter 5 mM solution required = 74.5513 × 5 / 1000 = 0.3728g NaCl

So, 0.3728g KCl was dissolved in small amount of de-ionized water and volume was made 1 liter by water.

c) Preparation of 150 mM BHT solution

Molecular weight of KCl = 220.35 g

1 liter 1 M solution required = 220.35 g BHT

1 liter 150 mM solution required = 220.35 × 150 / 1000 = 33.0525 g BHT

So, 3.305 BHT was dissolved in small amount of ethanol and volume was made 100 ml by ethanol.

d) Preparation of Phosphate buffer saline (pH 7.4) solution

Solution A = At first 0.2 M solution of Na_2HPO_4 was made by dissolving 28.39 g in 1 liter de-ionized water.

Solution B = 0.2 M NaH_2PO4 . 2 H_2O solution was made by dissolving 31.21 g in 1 liter de-ionized water.

Then, 40.5 ml of solution A and 9.5 ml of solution B was mixed together and diluted up to 100 ml by de-ionized water.

e) Preparation of Phosphate buffer saline (pH 6.6) solution

Solution A = At first 0.2 M solution of Na_2HPO_4 was made by dissolving 28.39 g in 1 liter de-ionized water.

Solution B = 0.2 M NaH_2PO4 . 2 H_2O solution was made by dissolving 31.21 g in 1 liter de-ionized water.

Then, 18.75 ml of solution A and 31.25 ml of solution B was mixed together and diluted up to 100 ml by de-ionized water.