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Screening, Characterization and Improvement of Nutrient Rich Okra Variety for Resistance to Yellow Vein Mosaic Virus

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University of Rajshahi

Ph.D. Thesis

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Thesis submitted for the degree of Doctor of Philosophy in the Institute of Biological Sciences Rajshahi University, Bangladesh.

Submitted By

KAZI ZAHIDUR RAHMAN, PhD. Fellow
SESSION: 2015-16, REG. NO: 0044
ROLL NO: 504

INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF RAJSHAHI
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Dedicated
To
My Departed Parents

DECLARATION

*I hereby declare that the whole work submitted as thesis entitled "**Screening, Characterization and Improvement of Nutrient Rich Okra Variety for Resistance to Yellow Vein Mosaic Virus**" in the Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh, for the degree of Doctor of Philosophy is the result of my own investigation carried out under the supervision of Professor **Dr. Md. Rezaul Karim-2**, Department of Biochemistry and Molecular Biology, Co-Supervisor Professor **Dr. Md. Anowar Hossain** and Professor **Dr. Mohammad Amirul Islam**, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh. The results of the investigation as embodied here are original and no part of the work referred to in the thesis has been submitted before as candidature for any other degree of this or any other university.*

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CERTIFICATE

*We hereby certify that the research work entitled "**Screening, Characterization and Improvement of Nutrient Rich Okra Variety for Resistance to Yellow Vein Mosaic Virus**" presented in this dissertation is based on the study carried out by **Kazi Zahidur Rahman**, Roll No. P-504 (Registration No: 0044, Session: 2015-2016) under our supervision in the Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh. It is suitable for submission to the style and contents for the partial fulfillment of the requirements for the degree of Doctor of Philosophy. The results of the investigation embodied here are original and have not been submitted before in substance for any other degree of this or any other university.*

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The Author

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LIST OF ABBREVIATIONS

C	: Control
S	: Standard
M1-M6	: Mutant 1st Generation to Mutant 6th Generation
M1L1-M6L7	: Mutant 1st Generation Line 1 to Mutant 6th Generation Line 7
RAPD	: Random Amplified Polymorphic DNA.
SSR	: Simple Sequence Repeat
YVMV	: Yellow Vein Mosaic Virus
ELCV	: Enation Leaf Curl Virus
DNA	: Di-oxy Ribonucleic Acid
gDNA	: Genomic DNA
PCR	: Polymerase Chain Reaction
AP-PCR	: Arbitrary Primed- Polymerase Chain Reaction
ISSR	: Inter-Simple Sequence Repeat
RFLP	: Restriction Fragment Length Polymorphism
AFLP	: Amplified Fragment Length Polymorphism
CAPS	: Cleaved Amplified Polymorphic Sequences
SCAR	: Sequence Characterized Amplified Region
SNP	: Single Nucleotide Polymorphism
STS	: Sequence-Tagged Site
EST	: Expressed Sequence tag
MAS	: Marker Assisted Selection
UPGMA	: Unweighted Pair Group Method with Arithmetic
PIC	: Polymorphic Information Content
kb	: Kilo base
bp	: base pair
QTL	: Quantitative Trait Loci
VNTR	: Variable Number of Tandem Repeat Sequence
SSLP	: Simple Sequence Length Polymorphism

SRAP	: Sequence Related Amplified Polymorphism
UV	: Ultra violate
CTAB	: Cetyltrimethyl ammonium bromide
EDTA	: Ethylene DiamineTetraacetic Acid
PVP	: Polyvinyl Pyrrolidone
RNA	: Ribonucleic Acid
OD	: Optical Density
TAE	: Tris-acetate EDTA
TE	: Tris-EDTA
cm	: Centimetre
nm	: Nanometre
µg	: Microgram
µl	: Microliter
mm	: Millimetre
min	: Minute
h	: Hour
Gen.	: Generation
SEM	: Standard Error of Mean
LD_{50}	: Dose required to kill 50% of test organism
DMSO	: Dimethyl sulfoxide
GAA	: Glacial Acetic Acid
TCA	: Trichloro Acetic Acid
IBPGR	: International Board for Plant Genetic Resources
BBS	: Bangladesh Bureau of Statistics
FAO	: Food and Agriculture Organization
USFDA	: United States Food and Drug Administration
CAD	: Coronary Artery Disease (CAD)
T2DM	: Type-2 Diabetes Mellitus
rpm	: Rotation per minute
FCR	: Folin-Ciocalteu reagent
TPC	: Total phenolic content

TFC	: Total flavonoid content
CA	: Catechin
CAE	: Catechin Equivalent
GA	: Gallic Acid
GAE	: Gallic Acid Equivalent
QU	: Quercetin
QUE	: Quercetin Equivalent
AA	: Ascorbic Acid
DNSA	: Dinitrosalicylic Acid
CONC.	: Concentration
mM	: Millimolar
NaN3	: Sodium Azide
IC	: Inhibitory Concentration
LC	: Lethal concentration
ROS	: Reactive Oxygen Species
TAC	: Total Antioxidant Capacity
FRAP	: Ferric Reducing Antioxidant Power
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
NO	: Nitric Oxide
ABTS	: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

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ABSTRACT

Okra [*Abelmoschus esculentus* (L.) Moench], is an important highly nutritive vegetable crop cultivated throughout the world. But the main constraint of okra production in Bangladesh is the viral disease caused by Yellow Vein Mosaic Virus (YVMV), which is spread by an insect vector namely white fly (*Bemisia tabaci)*. The loss in marketable yield has been estimated at 50-94 % depending upon the stage of crop growth at which the infection occurs. Although several local and commercial high yielding varieties are available, their pathogen resistance potentials are not enough for our local climatic condition. Moreover, the knowledge about the biochemical and nutritional composition of different varieties of okra cultivated in Bangladesh is very limited. Therefore, the aim of the study was to develop a nutrient rich and YVMV resistant high yielding okra variety. This study was undertaken to-evaluate the field performance of 10 varieties of okra against YVMV disease incidence, compare their nutritional, phytochemical and medicinal values and genetic improvement of nutrient rich variety through induced mutation technology for resistance to YVMV, molecular characterization of the YVMV resistant mutant lines by the development of molecular markers such as RAPD and SSR, identify the high yielding and YVMV resistant gene in mutant okra lines and finally the mutant lines are further re-investigated to analyse the nutritional (qualitative and quantitative) properties and cytotoxic level to confirm the higher nutrient content (nutrient rich establishment) subject to health issue. Ten varieties of okra e.g. Shamol Bangla (SB), Mahira Cross (MC), Orka Anamika Local (OAL), Shomy Hybrid (SH), Orka Anamika India (OAI), Shabuj Shathi (SS), Iron Bhendi (IB), BARI-1 (B1), Kolatia Bhendi (KB) and Dherosh Chamak (DC) were cultivated under the open field condition and premature pods were extracted using methanol as solvent. Qualitative and quantitative analyses of extracted phytochemicals were done using standard procedures. *In vitro* antioxidant activity of extracted phytochemicals was assayed by employing several methods. Cytotoxicity study of the extracts was performed using brine shrimp lethality bioassay. Several *in vitro* assay models were employed to investigate the medicinal properties of the extracts. Another way, okra premature pod extracts were prepared using water as solvent. Biochemical and nutritional analysis of

aqueous extracts were performed using standard procedures. Screened nutrient rich variety was used for mutation-breeding to develop YVMV resistant mutant okra lines. Seeds were treated with Sodium Azide (NaN_3) at concentrations of 1, 2, 3, 4 and 5 mM for chemical mutagenesis, sown under open field condition without any pesticide application and subsequent generations with YVMV resistance were screened starting from Mutant generation-1 (M1) to Mutant generation-6 (M6). Molecular characterization of nutrient rich and YVMV resistant mutant okra lines was performed by using RAPD and SSR markers. Genomic DNA (gDNA) of 7 mutant okra lines was extracted and purified by modified CTAB method, then amplified by PCR; RAPD and SSR bands were analyzed. Data were used for construction of phylogenetic tree by Dendrogram using UPGMA with Jaccard index of bioinformatics. Unique RAPD bands were cut and purified by DNA kit. YVMV resistant mutant okra Genes were Sequenced and identified by 1st BASE Co, Malaysia. Based on the field performance of selected varieties of okra F1 generation (parent), no variety was found to be resistant against YVMV. SB was found to be the most high yielding variety 520.78 g/plant (pod yield 17.35 t ha^{-1}) while with respect to viral resistance, OAI was found to be most tolerant variety (virus incidence 4.34%) followed by IB and B1. Analyses of phytochemical (qualitative and quantitative), nutritional and medicinal values were performed. Qualitative phytochemical analysis of pods methanolic crude extracts of 10 varieties of this plant successfully showed the presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins. In quantitative analysis- Out of twenty seven (27) parameters, Shamol Bangla (SB) was found to be the highest value in fifteen (15) parameters such as: Total phenol (11.37±0.39mg GAE/g), total flavonoid (9.88±0.09mg CAT/g), total flavonol (5.75±0.09mg QU/g), antioxidants: IC_{50} (µg/ml): 40.32±2.91(DPPH), 53.12±2.1(ABTS); TAC highest (1.285±0.013), FRAP highest (1.293±0.019), ash 10.647%, carbohydrate 7.42%, protein 2.15% (g%), vitamin-C 23.95, minerals: Ca 85.61 (mg per 100g), antibacterial activity (zone of inhibition 18 mm for 900 µg/disc), *in vitro* α-amylase inhibition 69.75% and α-glucosidase inhibition activity 73.48%. The above results suggested that SB is high yielding and nutrient rich variety among the selected varieties. Hence, SB was selected for mutation breeding to develop nutrient rich YVMV resistant new okra variety. NaN_3 at 4 mM concentration showed the highest yield 731.91 g/plant (24.39 t

ha^{-1}). Seven healthy virus free plants (M1) were selected and their seeds (M1 seeds) were collected for next season screening. M2 plants were grown under similar condition and Single Seed Population (SSP) method was used to screen the resistant lines. The results revealed that the selected lines (M2-M6) were resistant to YVMV. Among M2 lines, the yield of M2L5 was the highest 546.10 g/plant (18.20 t ha^{-1}). Among M3 lines, the yield of M3L5 was the highest 532.29 g/plant (17.74 t ha^{-1}) but less than the M2 plants. On the other hand, the yield M4L5 was the highest 588.52 g/plant (19.61 t ha^{-1}), which is higher than M3 plants. On the other hand, the yield of M5L5 was the highest 612.74 g/plant (20.42 t ha^{-1}) and was higher than M4 plants. In M6 mutant plants, the yield of M6L5 was the highest 693.48 g/plant (23.11 t ha^{-1}) and was higher than the M5 plants. Molecular characteristics of M5 okra lines such as M5L1, M5L2, M5L3, M5L4, M5L5, M5L6, M5L7 were compared with control C to study the genetic diversity using RAPD and SSR markers. gDNA was extracted and subjected to PCR amplification using 20 RAPD and 9 SSR primers. Out of 20 RAPD markers, 10 gave good amplification and scorable polymorphism and 2 gave monomorphic (unique band) band. A total of 40 RAPD fragments were formed by the 10 RAPD primers. Out of 40 bands, 32 were polymorphic. About 80% polymorphism was found among the 7 lines. Average number of bands per primer was 4.0 and average number of polymorphic bands per primer was 3.2. Average PIC value was calculated to be 0.415. Phylogenetic rooted tree showed C is very close to M5L3and far from M5L2. On the other hand, M5L5 & M5L1, M5L6 & M5L7, M5L4 & M5L2 were genetically in close relationship to each other. A total of 9 SSR primers were screened and all of them gave scorable amplification. The 9 SSR primers gave 16 bands and 14 of them were polymorphic. Number of bands per primer was calculated to be 1.77 and the number of polymorphic band per primer was 1.55. About.87.5% polymorphism was observed among individuals of okra lines. An average PIC value was 0.106. Phylogenetic tree showed C to be very close to M5L3 and far from M5L2. On the other hand, M5L5, M5L7 & M5L1, M5L6 & M5L4 genetic relationship was found very close to each other. It may be mentioned here that, in most of the cases, phylogenetic tree for RAPD and SSR markers showed similarities in morphological characteristics of the mutant okra lines. Among OPA- 9 and -11, OPA-11 showed unique band sizes of 1300, 800, and 400 bp for M5L5 line. Partial sequence homology of 800 bp with Internal Transcribed Spaces (ITS) ribosomal DNA of okra

was observed that might be responsible for demonstrating the mutated phenotype (YVMV resistance and high yield) of M5L5 okra. Re-checked study of Nutritional (Qualitative and Quantitative) properties and cytotoxic activity on Mutant M6 plants were performed using different methods (described in chapter 4). From the qualitative analysis of phytochemicals, it was observed that in most of the cases mutant lines showed similarities to each of other and M6L5 line was found same as Control (SB). In this study, the qualitative phytochemical analysis of pods methanolic crude extracts of mutant 7 lines with control of okra reconfirmed the presence of various phytochemical constituents as mentioned above. In quantitative analysis, the present study showed the highest nutrient values (nutrient rich) in all content of all lines compared to the previous study (Chapter 4) of SB values. On the other hand, out of 4 nutritional parameters 3 parameters were showed highest in M6L5 line such as: Carbohydrate 8.643 g%, protein 2.97 g% and vitamin-C 29.62 (mg/100g). In most of the cases, mutant okra values were also showed higher performance than control (SB).

In BSLT, it was observed and reconfirmed that the extracts exhibited brine shrimp low cytotoxicity (mild/slightly toxic) in a dose dependent manner (Table 7.3). The value of LC_{50}, indicates the low cytotoxic effect of okra pod extracts. Extracts lowest LC_{50} value of brine shrimp lethality was found in M6L1 842.62 µg/ml among the of mutant 7 lines with control of okra and Standard cytotoxic agent gallic acid was found to exhibit higher cytotoxicity with a lower LC_{50} value 13.45 µg/ml, maximum mortalities GA (100%) were observed at a concentration of 400 µg/ml. Extracts highest LC_{50} value of brine shrimp lethality was found in M6L2 1335.69 among the mutant lines of okra. The lethality activity (LC_{50}) of different lines extract was in the order of M6L1 (842.62) < C (890.74) < M6L5 (928.43) < M6L7 (934.58) < M6L3 (1034.53) < M6L6 (1035.83) < M6L4 (1135.23) < M6L2 (1335.69). The previous study control (SB) value of LC_{50} was 934.7 µg/ml. This study has successfully identified SB as the best among the cultivated varieties based on field performance against YVMV, nutrient contents, phytochemicals and medicinal values. Genetic improvement of nutrient rich (SB) variety of okra through induced mutation technology successfully achieved 7 lines of mutant okra (from the generation M1 to M6 plants) as the developed new variety which are resistant to YVMV. Molecular characterization of mutant okra 7 lines (M5) identified high yielding and YVMV

resistant gene in mutant okra line (M5L5). Identified partial gene sequences (M5L5) of molecular markers provide the knowledge about the mutation in the marker genes. Advanced okra 7 lines (M6) re-investigated subject to nutritional qualitative analysis showed the presence of same phytochemicals as control (SB). In quantitative analysis M6L5 line showed the higher nutrient content, as the nutrient rich best line and all the lines reconfirmed the nutrient rich variety resistant to YVMV. The analysis of cytotoxicity observed no significant change with control than the previous study (chapter 4) to health issue. Molecular characterization of mutant okra lines will help the plant breeders to develop new and improved cultivars with desirable characteristics. This study already characterized the 5^{th} generation of mutant okra lines as may be back crossed to identify the QTL or responsible gene for the resistance to YVMV disease and characterized the M6 generation of advanced lines as might be released as improved varieties in terms of YVMV resistance in future after successful field trials under seed certification authority.

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Introduction

Okra [*Abelmoschus esculentus* (L.) Moench], locally known as "Dherosh" is an important highly nutritive and economically important vegetable crop grown in tropical and sub-tropical and mediterranean parts of the world (Sanjeet *et al*., 2010; Haq *et al*., 2013). .It is a Dicotyledonae (along with such species as cotton, cocoa and hibiscus), belonging to the order Malvales and family Mallow (Schippers, 2000). It is valued for its edible green fruits. It is considered as the most used species of Malvaceae family. It is an annual or perennial herb growing upto 2 m height. The leaves are 10–20 centimetres long, broad and palmately with 5–7 lobes. The flowers are 4–8 cm in diameter with five white to yellow petals that have red or purple spot at the bases. The pollens are spherical with approximately 188 mi diameter. The fruit is a capsule up to 18 cm long with pentagonal cross-section containing numerous seeds (Okra-Wikipedia).

The name *okra* first used in 1670s came from a West African language, possibly Igbo *ọ́kụ̀rụ̀* (Douglas Harper, Inc. 2019). Igbo is officially recognized as one of the three major official languages in Nigeria. Igbo is written in the Latin script, which was introduced by British colonialists (Igbo_language; HarperCollins, 2019).

It is believed that okra mainly originated from the tropical part of Africa and is natural to West Africa (Purseglove, 1984). Several species from the genus *Abelmoschus* are grown and cultivated all over the world but in Asia, the most cultivated species are the *Abelmoschus esculentus* for its great nutritional and health benefits. World okra production (*A. esculentus and A. callei*) is estimated at 6 million ton/year (FAOSTAT, 2010). Okra is mostly grown in India, West Africa, South-East Asia, USA, Brazil, Australia and Turkey and is also a popular home-garden vegetable in many other areas. Gulsen *et al*. (2007) reported that the highest production of okra was in India

(70%) followed by Nigeria (15%), Pakistan (2%), Ghana (2%), Egypt (1.7%), and Iraq 1.7%. Young tender pods are a good source of vegetables and frequently used by many countries. The entire plant is edible and is also used to have several foods (Babu & Srinivasan, 1995; Madison, 2008; Lim, 2012; Jain *et al*., 2012; Maramag, 2013) and nonfood applications (Camciuc *et al*., 1998). In some African countries, leaves are consumed besides the young pods (Charrier, 1984) and different varieties of okra are also important for carrying medicinal properties (Velayudhan & Upadhyay, 1994).

In different regions of the world, okra is known by different names. It is also known as gombo in French, bhendi (Hindi), and bamiah (Arabic). Its name also varied within different regions of the world. It is known differently in South Asia as Okra, Okoro, Ochro, Ladies finger, Quingumbo, Gombo, Kacang Bendi Kopi Arab, Bhindi, Bamia, In middle east okra is known as Bamieh, Bamia, Bamya. In Southern USA it is named as Gumbo (Singha *et al*., 2014). People of Portugal and Angola called okra as Quiabo and in Cuba as "Quimbombo". Japanese people knew okra as Okura (Singha *et al*., 2014). Okra is delicious, tasty, gelatinous and tender, young tender pods of 3-5 days are usually used as vegetable, generally marketed in the fresh state. It has been reported that okra is a highly nutritional and medicinally important vegetable (Roy *et al*., 2014).

1.1.1 Taxonomy

Okra was earlier comprised in the genus Hibiscus, section *Abelmoschus* in the family Malvaceae, (Linnaeus, 1753). The section *Abelmoschus* was turn into eventually proposed to be raised to the rank of distinct genus via Medikus in 1787. Later in 1924 Hochreutinar reinstated the genus *Abelmoschus* of Medikus by proving that in Hibiscus the calyx, corolla, and stamens are separates whereas, in case of *Abelmoschus* calyx, corolla and stamens are fused together (Kundu & Biswas, 1973; Terrell & Winters, 1974). In spite of the genus *Abelmoschus* being originated from the Asiatic origin, *A. esculentus* has been found to be variable in India, West Africa, and tropical Asia. The extensive use of *Abelmoschus* was eventually accepted in the taxonomic and modern literature (Hochreutiner, 1924). Taxonomists have described 50 species in the genus *Abelmoschus*. Later it was fully documented by the taxonomical revision undertaken by van Borssum Waalkes and Bates (Borssum, 1966; Bates, 1968).

Kingdom	**Plantae**
Sub-kingdom	Viridiplantae
Infra kingdom	Streptopyta (land plants)
Super division	Embryophyta
Division	Tracheophyta (Trscheophytes)
Sub division	Spermatophytina
Class	Magnoliospsida
Super order	Rosanae
Order	Malvales
Family	Malvaceae (Mallows)
Genus	*Abelmoschus*
Species	*Abelmoschus esculentus*

- Binomial Name *Abelmoschus esculentus* (L.) Moench

1.1.2 Different species of *Abelmoschus*

An up-to-date classification of the genus *Abelmoschus* was accepted at the International Okra Workshop held at National Bureau of Plant Genetic Resources (NBPGR) in 1990 (IBPGR 1991) as given in Table 1.1

Table 1.1: Classification in the genus *Abelmoschus* accepted by IBPGR, 1991

Serial	Species
1	*A. moschatus* Medikus- subsp. *moschatus* var. *moschatus*- subsp. moschatus var. *betulifolius* (Mast) Hochr- subsp. *biakensis* (Hochr.) Borss. subsp. *tuberosus* (Span) Borss.
2	*A. manihot* (L.) Medikus- subsp. *tetraphyllus* (Roxb. ex Hornem.) Borss. var. *tetraphyllus* - var. *pungens*
3	*A. esculentus* (L.) Moench
4	*A. tuberculatus* Pal & Singh
5	*A. ficulneus* (L.) W & A.ex. Wight
6	*A. crinitus* Wall.
7	*A. angulosus* Wall. ex. W, & A.
8	*A. caillei* (A. Chev.) Stevels

(a) (b)

Figure 1.1: Healthy okra (*Abelmoschus esculentus)* plant flower (a) with fruits (b).

1.1.3 Varieties of Okra

There are many varieties of Okra (*Abelmoschus esculentus*) found in worldwide.

Some varieties of okra (Lal, 1997), such as:

- CLEMSON: The variety is dark green with angular pods. This okra takes less than two months to mature.
- EMERALD: This type variety is dark green with smooth round pods.
- LEE: The variety is a spineless type known by its deep bright green and very straight angular pods.
- ANNIE OAKLEY: This variety is a hybrid, spineless kind of okra with bright green, angular pods. It takes less than two months from seeding to maturity.
- CHINESE Okra: This is a dark green type grown in California and reaches 10 to 13 inches in length. These extra-long okra pods are sometimes called "ladyfingers."
- PURPLE Okra: It is a rare variety you may see at peak times. There is a version grown for its leaves that resemble sorrel in New Guinea.

Some other interesting okra varieties are included the following (gardenguides.com):

Red Burgundy, Silver Queen Okra, Star of David, Alabama Red, Cow Horn, Louisiana Short, Hill Country Heirloom Red, Burmese Okra and Jade okra.

It may be mentioned here that the both colours pod (green colour pod-Clemson spineless and red colour pod-Red Burgundy) okra flowers are white to yellow petals, often with a red or purple spot at the base of each petal. Red okra exhibits the same taste as the more popular green okra and differs only in fruit coloration. When cooked, the red okra pods turn green.

1.1.4 Origin and distribution

The centres of genetic origin of *Abelmoschus spp.* are West Africa, India, and Southeast Asia. In southern Asia, the wild and cultivated okra species show clear overlapping which is considered the centre of diversity. Another study shows that okra was originated first from somewhere nearby Ethiopia and the ancient Egyptians cultivated okra in the 12^{th} century B.C. Later the cultivation of okra was spread

throughout North Africa and the Middle East (Lamont, 1999; Siemonsma, 1982). The spread of the other species is the result of their introduction to America and Africa.

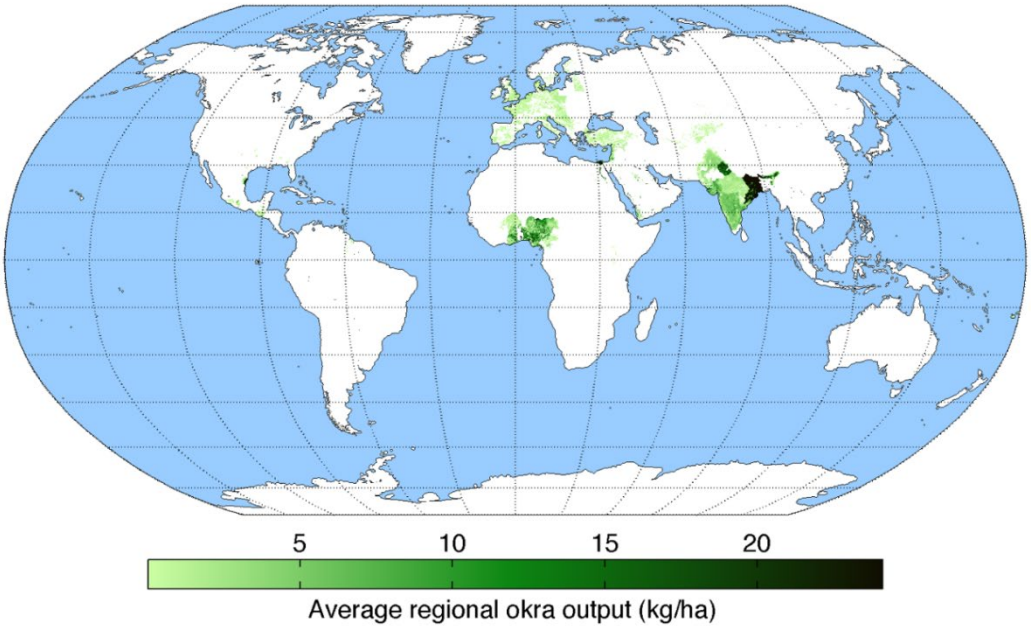

Figure 1.2: Distribution of okra all over the world (Adopted from Wikipedia).

1.1.5 Cultivation of okra

Okra is grown in many parts of the world, especially in tropical and sub-tropical countries for its fibrous fruits or pods containing round white seeds (Saifullah & Rabbani, 2009). This crop can be grown on a large commercial farm or as a garden crop (Rubatzky & Yamaguchi, 1997). Okra plants are grown commercially in many countries such as India, Japan, Turkey, Iran, Western Africa, Yugoslavia, Bangladesh, Afghanistan, Pakistan, Myanmar, Malaysia, Thailand, Brazil, Ethiopia, Cyprus and in the Southern United States (Qhureshi, 2007).

The warm temperature and humid wheather are preferred for faster growth and development of okra. It can be successfully grown in hot humid areas and in the temperature range 24 to 28°C. But at higher temperatures beyond 40~42°C, flowers may desiccate and drop causing yield losses. Higher seed germination rate of okra is found in the temperature range 24~35°C. Optimum seed germination occurs at 35°C.

Sandy to clay soils are preferred for okra cultivation. Relatively lighted, well-drained, relatively loose and friable well manured soil is good for okra cultivation. pH of the

soil should be 6.0-6.8 but the plant can tolerate soil with higher range of pH (5.5~8.0) (Jain *et al*., 2012). The plant prefers a soil with high potash content. Before seed sowing, the soil needs to plough properly. Most cultivars require about 4 months (120 days) from sowing before a crop is produced, though some early maturing varieties can produce a crop in 50 days in the tropic (Jain *et al*., 2012).

The highest productivity is reported from Egypt (12.5 tons/ha) followed by Saudi Arabia (13.3 tons/ha). The Area, Production and Productivity of Okra are given in Table 1.2

Table 1.2: Worldwide okra production & productivity

Country	**Area (´000ha)**	**Production (´000tons)**	**Productivity (mt/ha)**	**Share in world production (%)**
India	498.00	5784.00	11.60	73.25
Nigeria	455.10	955.60	2.10	12.10
Sudan	21.50	256.00	11.90	3.24
Iraq	19.53	151.22	7.70	1.92
Pakistan	14.60	113.20	7.80	1.43
Egypt	6.89	86.23	12.50	1.09
Ghana	21.10	82.50	3.90	1.04
Saudi Arabia	4.20	55.80	13.30	0.71
Cameroon	15.50	43.00	2.80	0.54
Others	44.53	243.71	5.50	3.09
Total	**1147.95**	**7896.26**	**6.90**	**100.00**

Source: From Indian Horticulture Database in 2011 (Kumar *et al*., 2011).

1.1.6 Production of okra in Bangladesh

Okra is cultivated all over the country as an important vegetable crop. In Bangladesh vegetable grows profoundly during summer season and rainy season. Most of the vegetables grow in winter season but okra grows well in summer and rainy seasons. So it can be a great benefit from okra over other vegetable crops. A report has shown that in 2009, 42000 tons of okra was produced from 10,122 hectare lands all over Bangladesh. Average production was 5.15 tons/hectare (BBS, 2010). This production

rate is not satisfactory compared to the production in other countries. There are several reasons for the low production of okra in our country.

Cultivation of low yielding and unimproved varieties is one of the major causes for low production. Again mismanagement of fertilizer and pesticides can also be a big reason for less production of okra. Another important reason is the disease incidence. Okra is highly prone to Yellow Vein Mosaic Virus and Enation Leaf Curl Virus. These two viruses cause severe yield loss in Bangladesh. Fungal attack is another cause for low production.

1.1.7 Biochemical constituents

Okra provides an important source of vitamins, calcium, potassium and other mineral matters which are often insufficient in the diet in developing countries (IBPGR, 1990). The properties of edible portion of okra is given in Table 1.3.

Table 1.3: Nutritional properties per 100 g of edible portion of okra.

Composition	**Amount (g)**	**Composition**	**Amount (mg)**
Carbohydrates	6.40	Vitamin C	13.10
Protein	1.90	Calcium	66.00
Calories	35.00	Magnesium	53.00
Fat	0.20	Oxalic acid	8.00
Moisture	89.60	Iron	0.35
Fibre	1.20	Potassium	103.00
Minerals	0.70	Riboflavin	0.01
Sulphur	30.00	Nictonic acid	0.06
Sodium	6.90	Thiamine	0.07
Phosphorus	56.00	Copper	0.19

The data are on the courtesy of IBPGR (1990).

1.1.8 Medicinal uses

Okra plant has important medicinal properties. It is a useful medicine for the treatment of haemorrhoids and ulcer (Adams, 1975). In Ayurveda, okra is used as an edible infusion and in different preparation for diuretic effect (Maramag, 2013). Okra is referred in the preparation of different herbal and traditional medicines. Okra mucilage can be used as a plasma replacement or blood volume expander (Savello *et al*., 1980; Markose *et al*., 1990; Lengsfeld *et al*., 2004; Adetuyi *et al*., 2008). It is also a good source of iodine which is useful in the prevention of simple goiter (Savello *et al*., 1980). Okra is useful in genitourinary disorders, spermatorrhoea and chronic dysentery (Singha *et al*., 2014). The leaves furnish an emollient poultice. It is used in the treatment of catarrhal infections, ardour urinae, diluvia and gonorrhoea. The roots are very rich in mucilage, having a strongly demulcent action. Okra root mucilage has almost the same chemical composition as that of medicinal plant common marshmallow *Althaea officinalis* (Tomoda *et al*., 1985). A report from China indicates that an alcohol extract of okra leaves can be useful for the elimination of oxygen free radicals, helpful in the improvement of kidney function, decrease protein urea, and remedy of intestinal disorders (Liu *et al*., 2005). Major uses of Okra as a medicinal plant are as follows:

1.1.8.1 Reduction of the risk of diabetes

Okra has hypoglycaemic effects due to its low carbohydrates and fiber contents. It helps reduce blood sugar level by regulating the sugar absorbing rate from intestinal tract (Sabitha *et al*., 2011; Karim *et al*., 2014).

1.1.8.2 Reduction of cholesterol level in blood

Okra helps to lower the serum cholesterol level and reduces the risk of heart diseases by binding with excess cholesterol and toxins in the bile acids, making it easy to eliminate and thus preventing many health problems (Wang *et al*., 2014). It contains soluble fibers and pectins. The soluble fiber of okra helps reduce the cholesterol level and therefor decrease the chance of cardiovascular disease. The pectin contents helps to lower cholesterol level by modifying the bile production through intestine (Gemede *et al*., 2014; Ngoc *et al*., 2008).

1.1.8.3 Digestive effects

Okra prevents constipation by helping in the digestive system. Insoluble fibers of okra keep the intestinal tract healthy. The mucilage and fiber contents of okra soaths and facilitates the elimination of the waste smoothly through the digestive tract thus preventing constipation. It is considered as a natural laxative (spillednews.com).

1.1.8.4 Drug delivery system effects

Okra may play a vital function in improved drug delivery system. Various reports applied okra polysaccharide as drug release agent. Okra gum as a mini-matrix for furosemide and diclofenac sodium tablets indicated prolonged release of furosemide and diclofenac sodium from the compressed tablets (Ofoefule & Chukwu, 2001).

1.1.8.5 Fight against cancer

Okra is a meaningful source of tumor-busting ingredients. A re-evaluate analysis of sixteen studies showed that folate may lower the risk of breast cancer (Chen *et al*., 2014). There is, in addition, another perk to okra's hefty amount of insoluble fiber, and it is a lower likelihood of rectal and colon cancer (dailyhealthvalley.com).

1.1.8.6 Improvement of vision

Vitamin A and β carotene are very important for the eye. Okra is considered as a good source of Vitamin A and β carotene. Thus it can be helpful for the improvement of the eyesight and can also help to prevent disease regarding eyes (whatthafact.com).

1.1.8.7 Treatment of stomach ulcer

Okra juice can actually help heal stomach ulcers. This is based on the fact that okra juice coats the surface of *Helicobacter pylori* bacteria making them unable to dock on the stomach walls (Lengsfeld *et al*., 2004).

1.1.8.8 Skin benefits

It can be used for the prevention of skin pigmentation, provides vitamin C for younger skin and dietary fiber for healthy skin (fabwoman.ng).

1.1.8.9 Hair benefits

Okra can be used as Scalp moisturizer and its mucilage contents can be used as hair conditioner. It can also use to get back bouncy and shiny hair (daily-bangladesh.com).

1.1.9 Other Uses:

A fiber acquired from the stems is applied as a substitute for Jute. It is also utilized in making paper and textiles (naturalmedicinalherbs.net). Mature fruits and stems containing crude fiber can also be applied for sacks and ropes (Watt, 1909), biogas and fuel (Dahiya & Vasudevan, 1987).

1.1.10 Health benefits

Okra is a vegetable rich in organic and inorganic nutrients that sustain human health and as feed for animals (Chattopadhyay *et al*., 2011; Ofoefule & Chukwu, 2001); Rahman *et al*., 2012; Wamanda, 2007; Siemonsma & Kouame, 2004; Saifullah & Rabbani, 2009). Okra is a very important vegetable crop which has been used for multiple purposes. Okra seeds contain good quality edible oil and high protein that are used to complement other protein sources. For baking bread with good technological and sensory characteristics, it is an effective and healthy food additive. The leaves and seeds are considered a valuable traditional medicine. Okra seeds contain abundant mineral elements, including iron, potassium, calcium, and manganese. It is also an oil and protein source, which can be used as a coffee additive (Çalışır *et al*., 2005). The antioxidant properties and vitamin C contents of okra help to promote healthy skin and blood. The soluble fibers help reduce the cholesterol level and therefore, decrease the chance of cardiovascular disease (Gemede *et al*., 2015). Okra is also useful for improving the eyesight. Okra pods can help to improve digestion, strengthens body immunity, control obesity, control asthma, packs a truck loads of nutrients, keeps from getting tired, prevents liver disease, reduce risks of deadly diseases like cancer specially beneficial for the control of colo-rectal cancer. The vegetable is a good provider of good bacteria called the probiotics. Probiotics helps in biosynthesis of vitamin B. They also help in proliferation just like the yoghurt (symptomsandcure. com). Young pods contain a significant amount of vitamin A and β carotene which are both very important for improving our eyes strength (Harris *et al*., 1997). Okra helps to reduce the blood sugar level by regulating the sugar absorbing rate from the

intestinal tract (Sabitha *et al*., 2011; Karim *et al*., 2014). Okra has several potential health beneficial effects on some of the important human diseases like cardiovascular disease, type 2 diabetes, digestive diseases and some cancers. Overall, Okra is an important vegetable crop with a diverse array of nutritional quality and potential health benefits (Ayushi *et al*., 2016). From the literature re-evaluate, it is evident that okra is a nutrient rich vegetable that has high-level medicinal values having the good health benefits.

1.1.11 Importance of the study

1.1.11.1 Field Performance Evaluation of 10 varieties of okra

To identify the disease (YVMV) resistant and high yielding variety, field performance is very much necessary.

Though okra has enormous economic benefits, it rarely reaches its maximum yield potential due to several constraints. Different factors are responsible for the low level of okra production in our country. The major limitation is the use of local and unimproved varieties; also high susceptibility to pest and disease incidence, and narrow genetic relationship existing varieties are some of the causes why our production is not increasing. Okra production plays a vital role in the economy of Bangladesh; so it is very important to take action for improving the production rate of this plant and also more attention should be paid in okra breeding program for development of new varieties with disease resistance as in the past no effort has been taken for its improvement (Eshiet & Brisibe, 2015). The causes of low productivity of okra are use of local unimproved cultivars and incidence of Yellow Vein Mosaic Virus (YVMV) disease (Sastry & Singh, 1975).

The cultivation of okra in Bangladesh is greatly challenged due to the severe incidence of Yellow Vein Mosaic Virus (YVMV) which spread mainly by whitefly (*Bemisia tabaci*). Yellow Vein Mosaic Virus (YVMV) is a devastating viral disease. The disease affects the quality of fruit and yield adversely. Frequent pickings, high operational cost, and residues of pesticides entering food chain are the limiting factors for chemical control of this disease.

In India, the occurrence of this disease was first reported by Kulkarni (1924) in Bombay province. The yield loss is about 50~94 % depending upon the infection on different stages of development in okra by the YVMV which causes veinal chlorosis. Severe chlorosis results in a complete yellowing of leaves, tubule destruction, plasmodesmata viral movement, early death of infected plant or yellowish malformed lower fruit yield and most of their pods are rotten before maturation (Kulkarni, 1924). Therefore, field performance evaluation is necessary for selection of the best high-yielding YVMV tolerant varieties.

Another cause for not increasing the average yield of okra in this country is the cultivation of low yielding varieties. Genotype with high yield potential and good characteristics is the basis of successful crop production and is important for increasing the productivity. Different genotypes of okra differ significantly from each other by different characters such as fruits number, length and mass of fruits and also fruit yield per plant. All these factors ultimately vary from one cultivar to another. A number of okra varieties are under cultivation in Bangladesh. Besides the improved cultural practices, there is a need to grow high yielding cultivars to increase the green fruit yield per plant and per unit area. Therefore, in the present study, 10 okra genotypes from local and exotic varieties were evaluated for their growth and yield potentiality against YVMV disease incidence as well as screened disease resistant germplasm.

1.1.11.2 Biochemical study

It is important to identify and determine the chemical compositions such as nutritional, phytochemical, biochemical and medicinal properties, which are very much effective for dietary requirements as well as prevention of various diseases.

Okra is a very important vegetable crop which has been used for multiple purposes. Different genotypes of okra differ significantly from each other by different biochemical characteristics such as nutrients, phytochemicals, organic and inorganic as well as medicinal compounds. A number of okra varieties are under cultivation in Bangladesh. Besides the improved cultural practices, there is a need to grow high nutritional (nutrient rich) quality and quantitative cultivars to ensure more health benefit including dietary requirements and safety from various diseases. In the

present study, 10 okra genotypes determined the nutritional quality and quantity of okra genotypes. Therefore, comparative study of biochemical, nutritional, phytochemical as well as medicinal values are necessary for selecting the best nutrient rich variety among the ten varieties of okra.

1.1.11.3 Mutation Breeding study

To develop the high yielding and nutrient rich resistant okra lines against YVMV disease mutation breeding technology is necessary.

A sudden and heritable change in a character of an organism is called mutation. Plasmagene mutations can easily be detected due to their cytoplasmic inheritance pattern. In crop species, many chromosomal mutations (especially those arising due to small changes in chromosome structure) would be classified as gene mutations. Gene mutations may themselves arise due to the replacement of one or more bases of a gene by another (base substitution), deletion or addition of one or more bases. The term mutation was first introduced by de Vries in 1900. The first variety developed through mutation was released in 1950 in Sweden; it was the Primax variety of white mustard (*Brassica hirta*). Mutation can be induced by mutagens. Mutagens are two types such as physical and chemical mutagens. The entire operation from treating the biological materials with a mutagen to the isolation of useful mutants is termed as mutation breeding. A mutant is an individual, which shows the mutant phenotype.
Hence, assessment of the potentialities of the existing cultivars is important because it depicts the genetic diversity of the base materials on which depends the promise for further development. Characterization of genetic resources, an essentially first step in any crop improvement programme, therefore, refers to the process by which accessions are identified, differentiated or distinguished according to their character or quality (traits) (Mish, 1991). Moreover, information carried on genetic relatedness among genetic resources of crop plants is useful, both for breeding and for the purposes of germplasm conservation (Brown *et al*., 1989).

For mutation breeding technology parent material is a prerequisite. In this study, genetic improvement of nutrient rich variety of okra through induced mutation technology has been attempted for YVMV resistant new okra variety.

1.1.11.4 Molecular characterization of mutant okra lines

Molecular characterization study of mutant okra lines will provide an opportunity for us to develop new and improved cultivars with desirable characteristics.

For the fulfillment of any crop improvement program the availability of genetic diversity is a needful (Haq *et al*., 2013). The value of a germplasm collection depends not only on the number of accessions it contains but also upon the diversity present in those accessions (Aladele *et al*., 2008). Relationship among okra germplasm and their genetic variability investigation may additionally play important function in plant breeding program to the biotic and abiotic stress tolerance (Gulsen *et al*., 2007). Genetic variability can be created by different mutation methods among individuals or it can naturally exist in the gene pool (Haq *et al*., 2013). Diversity based on morphological characters usually varies with environments and assessment of traits requires growing the plants to full maturity prior to identification of diverse genotypes. Now, the rapid development of biotechnology allows easy analysis of a large number of loci distributed throughout the genome of the plants. Molecular markers are now considered as an efficient and powerful tool in the study of genetic variability study among different closely related groups and in the elucidation of genetic relationships within these species (Chakravarthi & Naravaneni, 2006).

The genetic diversity of the plants can be analysed by using different molecular and morphological markers. Since DNA sequences are same in all the living cells of a plant regardless of physiological and developmental state of the tissues, it can be a great advantage to analyse the genome at the DNA level. Using of molecular markers in the assessment of genetic variation and finding genetic relationships among species have been proved to be a very powerful tool. In the field of molecular genetics use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most outstanding developments. To protect and use these plant genetic materials effectively, it is essential to develop markers that not only differentiate individuals and accessions, but also indicate the inherent diversity and relationships among collection territory (Ibrahim *et al*., 2010).

Different types of molecular markers having variation in their principles and methodologies are available such as AFLP, SSR, RFLP and RAPD for study the

genetic diversity (Kaur *et al*., 2013). Among all the several DNA based techniques, Random Amplified Polymorphic DNA (RAPD) is quite simple, less technology intensive, cheaper and it does not require any pre-sequencing for designing primers (Haq *et al*., 2013; Kaur *et al*., 2013). RAPD markers have been applied as a tool to estimate genetic diversity in a number of vegetable crops species like Triticum, Cotton, Okra, Radish, Capsicum, Onion and many other crops (Kaur *et al*., 2013). It has become very essential to identify individual varieties due to the number of genetically related varieties released by the way of breeders has made morphological identification more hard and the DNA fingerprints end up the genetic identity of a genotype. The goal of the study is to gain information about genetic diversity of mutant okra at molecular level. This information will be useful in the breeding programs for the development of new varieties of okra with desired agronomic traits such virus and fungus resistance.

1.1.11.5 Unique Band Purification and Identification

To identify the genetic variation in crop plants and their ancestors, also introgression between crops and their relatives need the identification of unique band (Soltis & Doyle, 2012). Finally, gene sequences of molecular markers will provide the knowledge about the mutation in the marker genes.

1.1.11.6 Re-investigation of Nutrients and Cytotoxicity

To determine and establish nutrient enrich mutant okra, reconfirmation study of nutrient is necessary to health issue.

1.2 Aims of the study

The okra accessions under cultivation, over the years in the various regions across the country are landraces. Nevertheless, these local varieties are facing different critical abiotic and biotic challenges such as high susceptibility to diseases and pests. In addition, these landraces have long maturity periods but short harvesting duration. They have poor nutritional quality, nonstandard in shape, color, and size, which make them unacceptable by consumers. Varieties those are high yielding and have early maturity with longer harvesting periods and resistant to viral, pest and other disease may be considered as ideal for the commercial cultivation. Improved varieties in

terms of fruit size, shape and colour are also very much desirable in the okra export market. For the detection and identification of okra with these desirable characters, this study is necessary. It helps us to assess qualitative and quantitative variations among collections of the okra landraces through morphological and genetic evaluation and thus exploit such variations in breeding programmes to develop improved and high yielding varieties.

- The main constraint of okra production in Bangladesh is the viral disease that caused by Yellow Vein Mosaic Virus (YVMV) which spread by an insect vector namely white fly (*Bemisia tabaci).*
- The loss in marketable yield has been estimated at 50-94 % depending upon the stage of crop growth at which the infection occurs. Although several local and commercial high yielding varieties are available, their resistant potentialities (tolerance levels) are not so promising for local climatic condition.
- Okra is nutritionally and medicinally important vegetables but the knowledge about the biochemical, nutritional composition, phytochemical and medicinal values of different varieties of okra cultivated in Bangladesh is very limited.

Keeping the above problems in mind, I have undertaken this study to identify and characterize the nutrient rich okra variety and applied the mutation breeding technology for developing YVMV resistant okra lines and characterized them at molecular levels.

Therefore, objectives of this experiment are as follows;

- ❖ Evaluation of field performance against YVMV disease incidence of ten selected varieties of okra under study.
- ❖ Comparative study of nutritional, phytochemical and medicinal values of those varieties of okra.
- ❖ Genetic improvement of nutrient rich variety of okra through induced mutation technology for resistant to Yellow Vein Mosaic Virus.
- ❖ Molecular characterization of nutrient rich and YVMV resistant mutant okra lines by the development of molecular markers such as RAPD and SSR.
- ❖ Identification of high yielding and YVMV resistant gene in mutant okra lines.
- ❖ Finally, the mutant lines were further investigated for nutritional (qualitative and quantitative) properties and cytotoxic level to confirm the higher nutrient content (nutrient rich establishment) subject to health issue.

1.3 Socio-economic importance of the study

- Diversity study in okra genetic resources will provide an opportunity for plant breeders to develop new and improved cultivars with desirable characteristics.
- This research will help plant breeders to produce both farmer-preferred traits (yield potential and small pods etc.) and breeders preferred traits (pest and disease resistance and photosensitivity, etc.)
- Identified partial gene sequences of molecular markers will provide the knowledge about the mutation in the marker genes.
- We believe that the information obtained in this research will be valuable to develop new varieties okra with high yield and disease resistant for future breeding programs.
- The research will play important roles in enhancing the food security of Bangladesh in future.

CHAPTER TWO
LITERATURE REVIEW

Okra cultivation in Bangladesh is a great challenge due to severe incidence of a disease caused by YVMV, a member of Geminiviridae family of Begomovirus genus. It is a ssDNA virus of nanogenome associated with a beta satellite, transmitted by the insect vector mainly White fly (*Bemisia tabaci*). This disease was first reported in 1924. It causes veinal chlorosis in severe cases resulting in complete yellowing of leaves, tubule destruction, plasmadesmatal viral movement, early death of infected plant or yellowish malformed lower fruit yield and most of their pods are rotten before maturation (Tripathi, 2013; Hull, 2009).

Sastry & Singh, (1975) identified the causes of low productivity of okra by the use of local unimproved cultivars and observed the incidence of YVMV disease. The loss in marketable yield has been estimated at 50-94 % depending upon the stage of crop growth at which the infection appears.

Gopalan et al. (2007) investigated the biochemical properties of okra. They determined that 100 g edible portion of okra fruit contains moisture 89.6 g, protein 1.9 g, fat 0.2 g, fibre 1.2 g, phosphorus 56.0 mg, sodium 6.9 mg, sulphur 30 mg, riboflavin 0.1 mg, oxalic acid 8 mg, minerals 0.7 g, carbohydrates 6.4 g, calcium 66 mg, iron 0.35 mg, potassium 103 mg, thiamine 0.07 mg, nictonic acid 0.6 mg, vitamin C 13 mg, magnesium 53 mg and copper 0.19 mg.

Akingbala *et al*. (2003) noted okra seeds to have appreciable protein content. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro- peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Soni *et al*., 2012). Alia *et al*. (2003) determined that antioxidants are powerful free radical scavengers in the body, while free radicals such as peroxide, hydroxyl radical, singlet oxygen etc. are highly

reactive chemical substances that travel around in the body and cause damage to the body cells.

Pytochemicals which are secondary metabolites of plants viz tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and phenols are responsible for their bioactivities such as antimicrobial, antidiabetic and antioxidant etc. (Lemos *et al*., 1990; Gaikwad *et al*., 2014 and Kahkonen *et al*., 1999). Phenolic compounds could serve as antioxidants against various diseases induced by ROS (Bhattacharya *et al*., 2012; Zhishen *et al*., 1990; Zheng *et al*., 2014 and Zhou *et al*., 2014). Food industries use several synthetic antioxidants which may be responsible for liver damage and carcinogenesis (Grice, 1988 and Witchi, 1986).

Okra is an important highly nutritive and medicinally important vegetable crop consumed throughout the world. Natural products are becoming the cynosure to inhibit and scavenge these reactive oxygen species (Alang *et al*., 2010). Liao *et al*., 2012 noted the highest amount of total phenolics and flavonoids in flower of okra.

Improvement of a specific crop species depends on an immense genetic diversity and extent to which the desirable characters are heritable. For a successful plant breeding program, assessment of genetic diversity is a prerequisite on which further line of action depends (Haq *et al*., 2013). Nowadays, most of the plant breeding programs are focused on conserving the agriculturally important crop species and up to now only a few agriculturally important varieties are studied for their genetic diversity. The goal of diversity study is to uncover the molecular markers of the economically important plants as well as the other types of plant species. Therefore, attempt has been taken for okra improvement by genetic diversity study at molecular level.

The idea of the genetic marker does not come suddenly; Mendel used phenotype based molecular marker system in the nineteenth century. Later the phenotype based marker of *Drosophila melanogaster* led to the establishment of the theory of genetic linkage (Waseem *et al*., 2012). Since then the genetic marker evolved from a morphological marker through cytological, biochemical to DNA markers. Cytological markers were used widely in the past but the unusual occurrences and laborious procedures make the cytological marker less popular. Biochemical and other protein-based markers are used mainly for the detection of a variance of amino acids and is

considered as the first set of molecular marker. Biochemical markers were popular before the uses of DNA markers. DNA markers detect the variance of the DNA sequences of individuals and these markers are not changeable with the environmental effects. They rely on the DNA sequences in contrasts to cytological and biochemical markers that rely on visible characters and traits and protein produced by genes respectively (Bekele & Bekele, 2014).

An ideal molecular marker should contain the following criteria:

1. Molecular marker should be reproducible and highly polymorphic.
2. It should be easily accessible (no cloning).
3. Must have an easy exchange of data between different laboratories.
4. Ideal molecular marker has selective neutral behaviour (no pleiotropic effect).
5. The assay should be easy and rapid.
6. Ideal molecular marker has co-dominant inheritance.
7. Development at a reasonable cost.
8. Occurrence at the genome should be frequent.

It is really complicated to find a molecular for particular character. Depending on the type of study to be attempted and on the lab facilities, a specific marker system can be selected. Different molecular marker systems differ from each other in respect to genomic abundances, locus specificity, strength of polymorphism, development cost of different marker system, requirements of DNA sequencing. Today various types of molecular marker are used to estimate genetic diversity of plants and are classified as hybridization-based molecular marker and the other is Polymerase Chain Reaction (PCR) based molecular marker. In the hybridization-based molecular marker system, genomic DNA is first digested with a restriction endonuclease and then hybridized with a labelled probe that is a DNA of known sequences. In PCR based molecular marker system DNA fragments are amplified in the thermal cycler using specific or arbitrary sequences (primer). Some primer is gene-specific and some primers are randomly selected. The amplified PCR products are visualized by electrophoresis or autoradiography. After electrophoresis, the products are viewed under the gel documentation system. PCR is extremely sensitive and it functions very rapidly. Hybridization-based molecular marker system is time-consuming and complicated in

their principle. For this reason, PCR based molecular marker is widely used in the recent years.

For continual maintenance and development of agricultural production, it is very important to conserve and study the plant genetic resources. All the conservation effort requires the identification and characterization of the diversity present in both gene pools and genBank. Description of morphological traits is the first instance for genetic diversity assessment program or conservation efforts. But morphological traits have some limitations in this sense that sometimes they are affected by the environmental factors or sometimes highly heritable traits shows little differences in the genetic level. Morphological traits cannot provide full genomic information. This limitation of morphological marker results in the development of biochemical, protein-based technology and other molecular marker systems that can differentiate polymorphism at the DNA level. However, characterization of morphological traits can't be replaced by either molecular or biochemical techniques. Molecular and biochemical techniques are considered as complementary to the morphological traits.

The information gained on different types of molecular marker techniques used in different plant species as well as in okra is re-evaluated here with a little emphasis on Random Amplified Polymorphic DNA and Simple Sequence Repeat marker.

2.1 Types of molecular marker

2.1.1 Morphological marker

Morphological markers for study plant genetic diversity analysis was first introduced in the molecular biology by Goethe in 1790 (McDonald *et al*., 2001). This is done by analysing some representative plants of the total population of an accession using some key features. Principal component analysis result will identify those features of the accession that is responsible for the diversity. Morphological characters build the central theme of any organism and they can be assessed, measured and counted (Heywood *et al*., 1967). Morphological markers can be selected for diversity analysis on the basis of availability, observation and their importance in identifying and characterizing of plants. Some of the characters are not affected by environmental factors and they are identified as the constant feature of that plants and other

characters are changed within different environmental conditions (Deborah *et al*., 1998). Their phenotypic expression occurs due to the combined effect of environmental factors and genotypic character of the plants.

Plant phenotypic characters are important for the study of plant genetic resources but there is some limitation to the morphological markers. The main limitation is the high dependency of these markers to the environmental factors (Stuber *et al*., 1999). This limitation of the morphological marker has led to the development of the molecular markers.

2.1.2 Biochemical markers

Isozyme markers were used for over 60 years for various application in biology such as to study genetic variability and diversity, to establish a phylogenetic relationship, to study taxonomy, population genetics, developmental biology and for characterization of plant genetic resources and in plant breeding's (Staub *et al*., 1996). Isozymes are a structurally different form of enzymes with same catalytic functions and usually originate from changes of amino acids causing differences in net charge or conformation of the enzyme or causing a change in their electrophoretic mobility (Kennedy & Thompson, 1991).

Enzymes are proteins consisting of amino acids which sometimes have a net charge depending on the stretch of amino acids. When a mutation occurs in the DNA it changes the amino acid comprising the protein thus changes the net charge of the amino acid that can be detected by protein gel electrophoresis. Because of the change in the electric charge, the migration rate of amino acid varies and different allelic variants can be detected which are called iso loci. Therefor allozyme marker is sometimes referred to as isozyme variation. Because of their consistency in the gene expression and less responsiveness to the environmental factors, allozyme markers were used extensively in the past for plant breeding and genetic diversity study.

The main advantage of the biochemical marker is their simplicity. DNA extraction and genome sequencing are not mandatory for analysis of biochemical marker. The assay is simple and quick. Allozyme markers are a co-dominant marker and highly reproducible. Some of the allozyme markers are very inexpensive depending on the

enzyme staining reagent used. Biochemical markers are the oldest of all the molecular marker-based analysis and are successfully applied in several crop improvement programs (Li, 1999).

Low level of polymorphism and low genomic abundances are the limitations of biochemical and allozyme markers. Biochemical markers are not genetic material and are products of gene expression; so they are susceptible to environmental factors (Glaszmann *et al*., 1989).

Ribozymes as a genetic marker has been utilized successfully in a number of crops like Avocado (Bailey, 1983; Torres & Bergh, 1980), *Citrus* (Torres *et al*., 1982), Pea (Weeden & Wolko, 1990), Cherimoya (Ellstrand & Lee, 1987) etc. Torkpo *et al*. (2006) used a biochemical marker for studying 22 accessions of okra in Ghana for diversity analysis in total protein content, total storage protein, asterisks.

2.1.3 DNA based molecular markers

DNA based molecular markers have modernized the way to assays variation among different germplasm. DNA based markers are highly reliable than any other type of molecular markers. Further, these markers are rapid, robust, and information may be obtained from a very little amount of plant genomic DNA and also these markers are not affected by the environmental factors. Different types of molecular markers are used for genetic diversity analysis of different species and they differ in their information content. Some of the most extensively applied molecular markers are described below:

2.1.3.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is the first and the most extensively applied hybridization-based DNA marker and was first used in the detection of DNA sequence polymorphism for gene mapping of a temperature sensitive adenovirus serotype (Grodzicker *et al*., 1974). This molecular marker is based on the pattern difference capability of the restriction enzyme. The basis of RFLP is that nucleotide change like insertion, deletion, substitution, inversion in the whole genome can create new restriction site (Yang *et al*., 2013). Genetic information is stored in the DNA sequences within the genotype and the basis of genetic variation

is the differences of this DNA sequence. Plants and animals are able to replicate this sequences with high accuracy and very rapidly but sometimes DNA sequences are changed by different physiological pathways or environmental factors. These changes can cause insertion, deletion, translocation, transposition, inversion or duplication of the sequences and these differences in the sequence may sometime cause the gain or loss of a fragment at the restriction site

Restriction fragments of different length can be recognized by using southern blot and a labelled probe. Though RFLP markers are not used widely, it has some importance in the identification of genetic diseases or to identify a person who is prone to disease and also use for the detection of the carrier for genetic disease (Ibrahim *et al*., 2010). RFLPs have also been applied to determine genetic diversity in several crops like Tomato, Pepper and Lettuce including taxa of Solanaceae, Poaceae, Fabaceae, Brassicaceae, Asteraceae and Linaceae. The advantages of RFLP analysis include high reproducibility, codominant inheritafnce and no sequencing of the genome is required. However, this is labour-intensive and time-consuming and includes the uses of hazardous radioactive isotopes and requires a large amount of purified DNA.

2.1.3.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is the first PCR based molecular marker system (Crossland *et al*., 1993). For its simplicity, it is widely used in place of RFLP. In this technique, short oligonucleotide sequences (10 bases long) are utilized for the amplification of nanogram amount of genomic DNA under low annealing temperature by PCR. Decamer primers are readily available from different companies (Sen *et al*., 2010). RAPD primer binds at different binding sites of the DNA but it is not certain exactly where the primer bind. This property has made the RAPD popular for analysis of genetic diversity of unstudied species in which sequence data are unavailable. Fragments are detected and used for the analysis of genetic diversity. The amplified fragments are usually 0.5-5 kb size range and are separated by agarose gel electrophoresis for the detection of polymorphism (Sen *et al*., 2010). RAPD marker is now widely used all over the world for the identification of accessions, plant breeding's and genetic diversity analysis (Fukuoka *et al*., 1992).

Major advantages of RAPD technique over other molecular markers are: it requires a very low amount of DNA for a single reaction, it is less technology intensive, free from using hazardous radioactive compound and it does not require sequencing of the DNA. However, this marker is not always reproducible and RAPD is not a locus-specific marker.

Haq *et al*. (2013) assayed genetic variability of 39 okra accessions using 20 polymorphic Random Amplified Polymorphic DNA. Among the 39 okra genotypes, 96% fragments were found to be polymorphic. Seven main clusters were placed by UPGMA cluster analysis. Maximum similarity (83%) was found between two accessions sabzpari 2001, Acc No 019221 and minimum similarity was found between two accessions Punjab selection and Acc No 019217.

Prakash *et al*. (2011) investigated the genetic relationship among 44 okra genotypes. A total of 14 RAPD primers were applied in the experiment, which formed 104 RAPD fragments where 74.03% of the fragments were polymorphic. The Average number of bands per primer was 7.41. RAPD data was used to calculate the squared Euclidian Distance Matrix and cluster analysis was performed using minimum distance variance.

Kaur *et al*. (2013) used 40 Random RAPD primers for genetic variability among 70 okra genotypes and 8 quantitive traits were used in the experiment.

Samarajeewa & Rathnayaka, (2004) sudied the genetic diversity of eight different okra accessions by 10 Random Amplified Polymorphic DNA. Total 130 bands were recorded for all eight genotypes where polymorphic bands, unique bands and monomorphic bands were 88, 08 and 34 respectively. These data were used to produce dendrogram for phylogenetic study among the different okra germplasms and a sub group suggests that they are genetically more related to each other.

Martinello *et al*. (2000) investigated genetic variability among 39 okra genotypes at a phenotypic level for highly stable and heritable morphological characters. 31 random decamer primers were employed for the study. A total of 103 RAPD fragments were generated. Dendrogram was produced by using the RAPD data for genetic variability study. RAPD technique has been made use in a number of crops by number of

research groups, like, Celery (Yang & Quiros, 1993), Barley (Tinker et al., 1993), Papaya (Stiles et al., 1993), *Brassica oleracea* L. (Dos Santos et al., 1994) etc.

2.1.3.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is a very recent DNA fingerprinting technique which was introduced by a Dutch company keygen to overcome the reproducibility problem of RAPD marker (Vos *et al*., 1995). AFLP marker has the widest application in the genetic diversity and in the study of population structure and differentiation. This method is based on the PCR amplification of the selected restriction digested fragment of the full genome. It combines the power of RFLP and RAPD to ligate fragments of any DNA regardless of its source and without any prior knowledge of its genome sequence and the analysis of AFLP is also very easy because polymorphism is detected by the absence or presence of bands rather than mapping the size of a different locus (Mondini *et al*., 2009). AFLP is an extremely sensitive method and the use of the fluorescent primer for automatic fragment detection and the software packages to analyse the fragments makes it really convenient for use in the population diversity analysis. Generally, 75-150 fragments are produced for each primer combination and each represents a unique primer binding sites (Farooq & Azam, 2002). AFLP markers are popular all over the world for use in population genetics, systematic, pathotyping and Quantitative Trait Loci (QTL) mapping (Mueller & Wolfenbarger, 1999).

This method generates a large number of fragments and can be used to detect genetic variation among individuals. AFLP has been successfully applied for diversity analysis in certain plants like the Common bean (Adam Blondon *et al*., 1994; Melotto *et al*., 1996) and other crops like Tomato (Ohmori et al., 1996) and Strawberry (Haymes et al., 2000) etc. AFLP analysis is useful for DNA fingerprinting (Hongtrakul et al., 1997) and also for mapping and cloning.

Moughan *et al*. (1996) reported the power of AFLP marker for the detection of genetic loci in different soybean cultivars with minimal cost and lower time requirement. Lombard *et al*. (2000) reported AFLP marker based genetic identification in repressed using cluster analysis and principal component analysis.

The AFLP marker can be assessed further by SCAR (Sequence Characterized Amplified Region) marker for obtaining band such as RAPD marker. This makes the AFLP marker more acceptable and has multiple functioning molecular marker technique. Martin *et al*. (1991) and Michelmore *et al*. (1991) initiated SCAR technique where the RAPD and AFLP techniques are further sequenced and comparatively larger primer (22-24) are designed for the amplification of specific locus of interest.

Salameh *et al*. (2014) assessed genetic diversity among 48 genotypes of Okra accession using 8 Amplified Fragment Length Polymorphism markers. The 8 selected primer pairs produced 150 polymorphic AFLP fragments. Based on this data UPGMA cluster analysis divided these 48 genotypes into 6 clusters on the basis of the Dice similarity coefficient.

2.1.3.4 Simple Sequence Repeat (SSR)

Microsatellite or Simple Sequence Repeats are referred as Variable Number of Tandem Repeat Sequence (VNTR) and Simple Sequence Length Polymorphism (SSLP) are widely dispersed in the eukaryotic genome and their polymorphism is the result of variation in the tandem repeat sequence of the core sequence (Wan *et al*., 2004). Microsatellites are a short stretch of DNA of which one to six bases can repeat over five to hundred times at each locus. Microsatellites occur not only in the nuclear genome but also in the mitochondrial and chloroplast genome as a repetition of guanine and cytosine (Jarne & Lagoda, 1996). It is possible to isolate microsatellite from any target species as one hundred five microsatellite loci are present in the genome. Microsatellites are found in the genome as co-dominant Mendallion pattern and can reveal as homozygote and heterozygote in each individual. Microsatellite markers are used to identify structure, classification, discrimination, the relationship in both individual and population relationships (Jarne & Lagoda, 1996).

One of the major advantages of using microsatellite marker is that these markers are locus-specific and highly polymorphic. SSR markers are co-dominant marker which allows this marker to distinguish between homozygotes and heterozygotes individual. Microsatellite markers are information rich and more variable than RFLP, AFLP and RAPD markers and a very low amount of DNA is required for SSR analysis.

The main drawback of microsatellite is the high development cost of microsatellite marker. It is also labour-intensive and time-consuming procedure involving construction and screening for microsatellite region in the whole genome and optimization of the primer. This has limited the use of microsatellite marker in economically less important crops plant. Fortunately, in some cases, the microsatellite marker developed for one plant is applicable to closely related plants.

Kumar *et al*. (2017) investigate genetic diversity in 69 germplasm of okra collected from different parts of India. A total of 19 microsatellite primer pairs were used and only 10 of them give good amplification and clearly scorable fragment. 24 distinct bands were produced with an average of 2.4 bands per primer. An average Polymorphic Information Content (PIC) was 0.729 and Resolving power was 1.841. Genetic similarity among okra germplasm was found 0.772. The lower level of genetic diversity was found on the basis of microsatellite marker. UPGMA cluster analysis showed three main clusters and some of the genotypes were found to be similar at the molecular level.

Kumar *et al*. (2017) conducted an investigation to study genetic variation among 96 different okra accessions and 94 of them were *A. esculentus* and one accession in each of *A. tuberculatus*, *A. moschatus*, *A. moschatus* subspecies tuberosus and *A. manihot*. A total 40 SSR primer pairs were screened and 30 of them give clearly scorable SSR fragments and these were used for further analysis. Total 213 SSR fragments was produced with an average of 7.13 bands per primer and 60.66% of them were found to be polymorphic. PIC value was recorded 0.52 indicating that the primers are informative. UPGMA cluster analysis grouped these genotypes into three main clusters. An accession named GAO-5 was found to be most diverse.

Singh *et al*. (2017) studied morphological and molecular-based diversity among 50 different genotypes of okra and 30 SSR primer pairs were used to study genetic diversity, marker index, resolving power, similarity index and phylogenetic study for genetic variation analysis. Similarity value for all the 50 genotypes of okra varied from 0.573 to 0.984. The lowest similarity was revealed by VRO-6 and PK highest. Out of these samples analysed, genotypes FB-10 and Azad bhindi-1 displayed the greatest genetic similarity, with a similarity coefficient value of 0.984. All genotypes

were distributed into nine distinct clusters. Cluster 3 was found to be the largest cluster containing 8 genotypes.

Sharma *et al*. (2015) analysed genetic diversity among 24 sweet and wild cherry types in Czech Republic. They have used 16 SSR primers for screening and comparison of sweet Cherry germplasm collections. They found all the SSR polymorphic and total 70 SSR alleles were produced with an average of 4.4 alleles per primer. Primer UDP-98-412 gives the highest number of polymorphic bands. They observed 10% unique alleles at different loci. Average PIC value was calculated as 0.523. Cluster analysis separates the germplasms into two clusters. High level of genetic diversity was mentioned among the germplasm of cherry.

2.1.3.5 Inter-Simple Sequence Repeat (ISSR)

Inter-Simple Sequence Repeats are DNA fragments of 100-3000 bp long adjacent between two identical microsatellite region in the opposite orientation. It uses the single microsatellite primer to amplify mainly an inter-simple sequence of different sizes. ISSR markers are very simple and randomly distributed in the genome and use of radioactivity is not essential for this marker. They require a very low amount of DNA and exhibits a dominant inheritance pattern. The primer used in ISSR is usually longer (15-30) which gives it higher annealing temperature and higher strength (Joshi *et al*., 2000). The amplified PCR product is generally 200-2000 bp long and can be viewed on both agarose gel and polyacrylamide gel through silver staining. Though ISSR has the characteristics of microsatellite it does not require any prior knowledge of the genome of interest. ISSR markers are used for gene mapping, identification of variables, genetic diversity analysis, taxonomy etc (Nilkanta *et al*., 2017). This molecular marker system is quick, less technology intensive and cheap also. It does not require pre sequencing of the genome and a very low amount of purified genomic DNA is required for a single assay. Like as the RAPD, ISSR also has reproducibility problem. Sometimes the level of polymorphism varies with the detection method. Usually it shows better polymorphism in polyacrylamide gel than in the agarose gel. Co-migration of the fragments from the non-homologous genome is another limitation of ISSR.

Huang *et al*. (2017) performed genetic diversity and evolutionary analysis among 34 accessions of okra by using ISSR primers. A total of 128 repeatable bands were observed and of them, 90 bands were found to be polymorphic. Percentages of the polymorphic band were 70.3% with an average of 7.6% per primer. The Phylogenetic tree was constructed using all the bands produced. By UPGMA cluster analysis 34 accessions of okra were broadly classified into two major groups. They also confirmed this conclusion by two-dimensional matrix. The PIC values were varied from 0.4507-0.6801 indicating that the primer used was informative and authentic.

2.2 Variability study in okra

For plant breedings and crop improvement program, information on the genetic level is very important. Very few works have been reported on okra in Bangladesh. Knowledge on the genetic diversity among different varieties of okra will help plant breeders to improve the quality of the fruit and also to develop new varieties with improved characteristics. Inter-specific hybridization is possible between *Abelmoschus spp.* (Hamon & Hamon, 1991). This will increase diversity among okra genotypes and will accelerate the okra breeding program. Martin & Rhodes *et al*. (1983) reported that *A. esculentus* species differ from *A. caillei* based on seeds characteristics like seed color, weight, oil contents etc.

Molecular marker has become a useful tool for analysis relation and diversity among different accession either local or wild-type. Molecular markers are not affected by the environmental factors thus give a reliable result on plant genotype. Studies of the molecular marker in okra are lagged behind compared to any other economically important crop plants.

Gulsen *et al*. (2007) used a phenotypic marker and Sequence Related Amplified Polymorphism (SRAP) marker to estimate genetic diversity and relationship among 23 different varieties of okra. 39 combinations of forward and reverse primers were used against 21 Turkish okra genotype and 2 randomly selected USA genotypes. Total 97 fragments were produced of which 50% were found polymorphic to all the genotypes. 74% of the total genotypes were found distinguished from the others.

Saifullah *et al*. (2010) investigated molecular phylogeny and genetic diversity among one hundred twenty one okra genotype by Random Amplified Polymorphic DNA marker. Out of 39 RAPD primers, 5 primers gave 38 clearly scorable RAPD fragments. 32 bands were found to be polymorphic. Average band per primer was found 7.6 bands per primer. All the genotypes were divided into 8 distinct clusters. Correlation value was found 0.54 based on morphological and molecular data. A high genomic diversity of the tested germplasm was found in the experiment.

Nwangburuka *et al*. (2011) used 29 okra accession collected from different parts of Nigeria for assessment of genetic diversity. RAPD markers were used in the study. Patel *et al*. (2018) used RAPD and SSR markers for analysis of genetic diversity for Yellow Vein Mosaic Virus among ten different okra genotypes. 30 RAPD primers were used and 13 of them gave clearly scorable RAPD fragments. These 13 RAPD primers shape 228 loci of which 224 loci were found polymorphic with 93.36% polymorphism. 4 SSR primers produced a total of 60 bands with average 0.72 PIC value. On the idea of clustering pattern, RAPD markers are useful for genetic diversity even as SSR markers from cross-species are not useful for varietal screening of okra against YVMV.

CHAPTER THREE
FIELD PERFORMANCE EVALUATION OF SELECTED OKRA VARIETIES

3.1 Introduction

Okra (*Abelmoschus esculentus* L.) is considered an important vegetable crop cultivated throughout Bangladesh. Okra is delicious, tasty, gelatinous and tender; fruits are used as a vegetable. Young tender pods of 3-5 days are usually treated as vegetable, generally marketed in the fresh condition. In Bangladesh vegetable grows profoundly during summer and winter seasons. Most of the vegetables grow in winter season but okra grows well in summer. Okra production in Bangladesh is mostly during February- July (Rashid, 1999). So this can be a great benefit of okra over other vegetable crops. World production of okra (*A. esculentus* and *A. callei*) as fresh fruit vegetable is measured at six million ton per year (FAOSTAT, 2010). However, production of okra in Bangladesh is not satisfactory. A report has shown that in 2009, 42000 tons of okra was produced from 10,122 hectare lands all over Bangladesh. Average production is 5.15 tons/hectare (BBS, 2010). This production rate is not satisfactory compared to the production in other countries. There are several reasons for the low production of okra in our country.

Cultivation of low yielding and unimproved varieties is one of the major causes for low production (Sastry & Singh, 1975). Again mismanagement of fertilizer and pesticides can also be a big reason for less production of okra. Another important reason is the disease incidence (Sastry & Singh, 1975). The main constraint of okra production in Bangladesh is the viral diseases caused by Yellow Vein Mosaic Virus (YVMV) and Enation Leaf Curl Virus (ELCV) which spread by an insect vector namely white fly (*Bemisia tabaci*). These two virues cause severe yield loss in Bangladesh. The loss in sellable yield has been estimated at 50-94 % depending upon the stage of crop growth at which the infection arises. Fungal attack is another prominent cause for low production. Although several local and commercial high

yielding varieties are available, their resistant potentialities (tolerance levels) are not so promising for local climatic condition. So, field performance evaluation is necessary to identify the best virus tolerant varieties.

Another cause for not increasing the average yield of okra in this country is the cultivation of low yielding varieties. Genotype with high yield potential and good characteristics is the basis of successful crop production and is important for increasing the productivity. Different genotypes of okra differ significantly from each other by different phenotypic characters like fruits number, length and mass of fruits and also fruit yield per plant. All these factors ultimately vary from one cultivar to another. A number of okra varieties are under cultivation in Bangladesh. Besides the advanced cultural practices, there is a need to expand extra excessive yielding (high yielding) cultivars to increase the tender fruit yield per plant and per unit area. In the present study, 10 okra genotypes from local and exotic varieties were evaluated for their growth and yield potentiality against YVMV disease incidence as well as screened disease resistant germplasm.

3.2 Materials and Methods

3.2.1 Experimental site

The experiment was conducted in the Biochemistry Research Garden, University of Rajshahi, Bangladesh

3.2.2 Collection of Plant materials

Seeds of 10 selected varieties of okra were collected from different vendors of different parts of Bangladesh as listed bellow in Table 3.1.

Table 3.1: Sources of ten varieties of okra selected for cultivation in this study.

Name of variety	Abbreviation	Sources/Vendors
Shamol Bangla	**SB**	Metal Agro Limited, PBL Tower (14th Floor), 17 North Commercial Area, Gulshan Circle Dhaka, Bangladesh.
Mahira Cross	**MC**	M/s. Rajshahi Beej Bhandar, Horogram Bazar, Court Station Road, Rajshahi, Bangladesh.
Orka Anamika Local	**OAL**	Shabji Bijagar, Jamal Gong Road, Joypurhat, Bangladesh.
Shomy Hybrid	**SH**	Katakhali Bazar, Rajshahi, Bangladesh.
Orka Anamika India	**OAI**	From India on 28.01.2016
Sobuj Sathi	**SS**	Momin Seed Company, 145-Siddik Bazar, Dhaka-1000, Bangladesh.
Iron Bhendi	**IB**	M/S. Bikrampur Seed Co. 145-Siddik Bazar, Dhaka-1000, Bangladesh.
BARI-1 (Local)	**B-1**	Menha Seed Company, 145-Siddik Bazar, Dhaka-1000, Bangladesh.
Kolatia Bhendi	**KB**	Khalak Seed Company, 174-Siddik Bazar, hannan Mansion, Dhaka-1000, Bangladesh.
Dherosh Chamak	**DC**	Shabji Bijagar, Jamal Gong Road, Joypurhat, Bangladesh. Collected from: Haque Seed Store, Nawdapara (under the janata bank), Sapura, Rajshahi, Bangladesh.

3.2.3 Methodology

3.2.3.1 Okra cultivation: The colected okra seeds as described in the Table 3.1 were stored in air-tight containers in cool, dark and dry place. Land was prepared as plots: each having: 3 m×1 m dimension and space between plants: 30 cm × 40 cm; and number of seeds: were 30. Forty five centimeters were left for irrigation and drainage between two beds. Manures and fertilizers were used as recommended by Bangladesh Agricultural Research Institute (Mondal *et al*., 2011). No pesticide was applied during the experimental studies. Seed soaked in distilled water for 24 h. Germinated seeds were sown in 30 plots with Randomized Complete Block Design (RCBD) with 3 replicas of each variety. Plots were tagged by the name of variety with seed sowing

date. Okra plants were cultivated in the open field condition and maintained properly with irrigation and weeds cleaning.

3.2.3.2 Data collection

Data on morphological trait as listed below were recorded every two weeks during the period of cultivation over 120 days. Data were collected from 30 plants of each variety from each plot.

Morphological traits -

1. Plant height (cm)
2. Number of branches
3. Number of leaves
4. Number of flowers
5. Number of fruits
6. Fruit weight (g)
7. Number of seeds per fruit
8. The weight of 100 seeds (g)
9. Number of Yellow Vein Mosaic Virus infected plants (%)
10. Yield per plant (g) and total pod yield (t ha^{-1})
11. Biomass per plant (kg)

Field performance evaluated by the incidence of virus was calculated and grading was given as described by Sharma & Sharma (1984) as follows:

Grade	Percentage of viral disease incidence	Category
Resistant (R)	0.0	IV
Tolerant (T)	1~30	III
Susceptible (S)	31~70	II
Highly Susceptible (HS)	71~100	I

The materials and methodology is summarized in the flow diagram below.

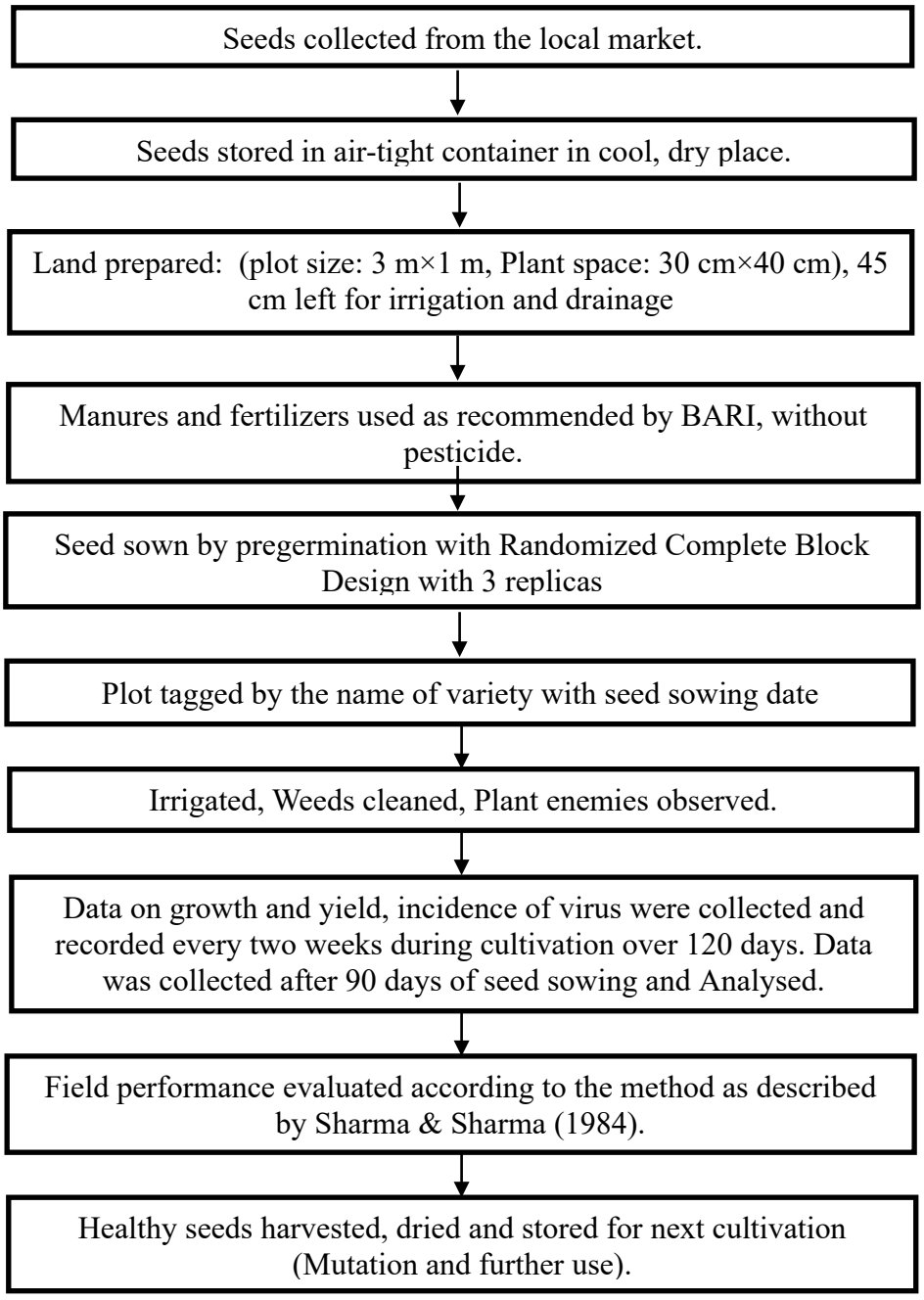

Figure 3.1: Flow diagram showing the okra cultivation for field performance evaluation.

3.3 Results and Discussion

Field performance of the cultivated okra was evaluated based on their morphological characters. Data were collected and mean values were calculated as given in Table 3.2.

i) Plant height (cm)

In case of plant height, Shamol Bangla was found as the tallest (106.55 cm) variety followed by Mahira cross (95.67 cm), Kolatia Bhendi (91.23 cm) and Orka Anamika Local (86.68 cm). BARI-1 was found as the shortest variety (78.55 cm) among all the okra genotypes. Plants of Iron Bhendi were also very short (79.59 cm).

ii) Number of branches per plant

Branch number was higher in Iron Bhendi (4.00) than any other varieties. Shomy Hybrid (3.66), Sobuj Sathi (3.66), Kolatia Bendi (3.66) and Dherosh Chamak (3.66) also showed high branch number. The lowest branching was found in Shamol Bangla (3.00).

iii) Number of leaves per plant

The highest leave number was seen in Orka Anamika India (36.66) followed by Orka Anamika Local (35.66), Shamol Bangla (35.33) and Kolatia Bhendi (35.33). The lowest number of leaves was counted in Sobuj Sathi (32.33) follwed by Iron Bhendi (32.66) and Mahira Cross (32.66).

iv) Number of flowers per plant

Flowers per plant were also counted in the present study and average number of flowers per plant was found high in Shamol bangla (1.66). The lowest flower number (1.00) was shown by Mahira Cross, Orka Anamika Local, Shomy Hybrid and Sobuj Sathi.

Figure 3.2: Experimental garden showing okra cultivation and disease manifestation.

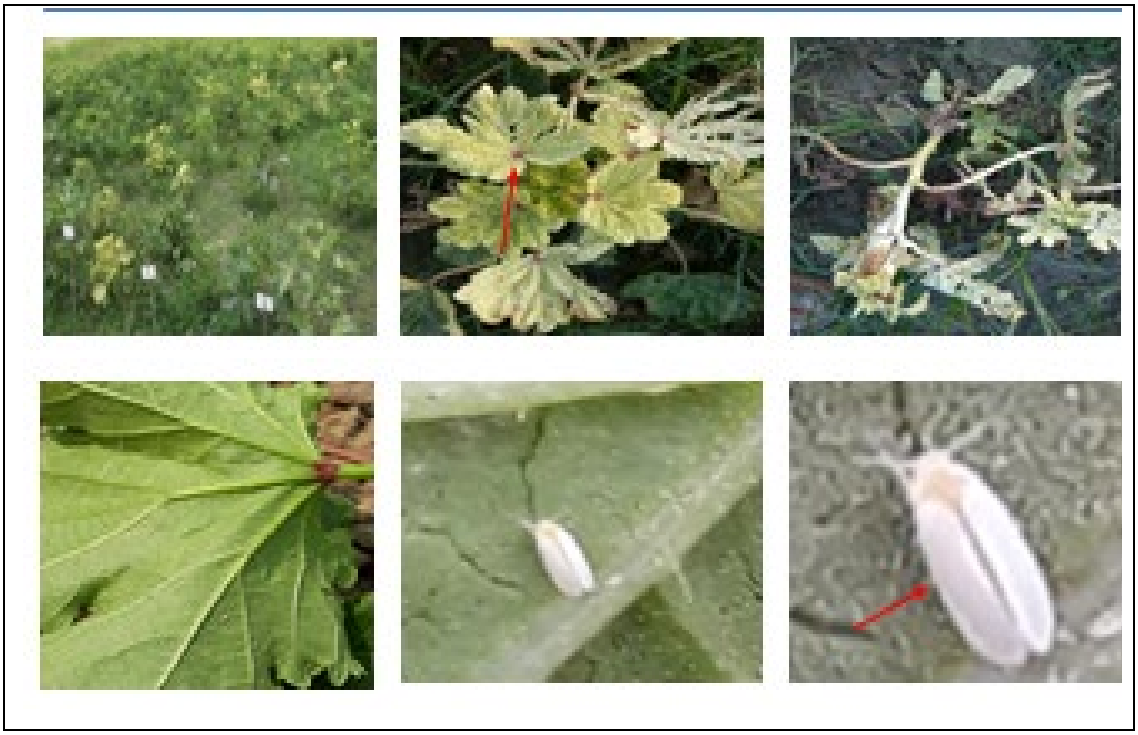

Figure 3.3: Mode of attack of the vector white fly and effect of viral disease on morphological changes of okra plant.

v) Number of fruits per plant

Fruits number per plant determines the overall yield of that plant. The highest fruit number was recorded in Shamol Bangla (25.00) followed by BARI-1 (22.33), Mahira Cross (21.66), Sobuj Sathi (21.66) and Orka Anamika India (21.00). The lowest fruit number was counted in Kolatia Bhendi (19.33). Fruit number was low in Iron Bhendi (20.00), Orka Anamika Local (20.33), Shomy Hybrid (20.33) and Dherosh Chamak (20.33).

vi) Weight/fruit (g)

Higher weight of fruit means larger size of fruit. Shamol Bangla was recorded the highest fruit weight (20.83 g). Fruit weight was also found very high in BARI-1 (19.07 g), Sobuj Sathi (18.16 g), Mahira Cross (17.95 g) and Orka Anamika India (17.55 g). On the other hand, the lowest weight of Kolatia Bhendi fruit was 15.99 g/fruit. Iron Bhendi (16.62 g), Shomy Hybrid (16.82 g), Dherosh Chamak (16.85 g) and Orka Anamika Local (16.88 g) also showed low fruit weight.

vii) Number of seeds per fruit

As the fruit size of the Shamol Bangla was largest, number of seeds was also found the highest (77.66) followed by BARI-1 (64.33), Mahira Cross (63.66), Sobuj Sathi (61.66) and Orka Anamika Local (61). The lowest number of seeds was counted in Shomy Hybrid (50.66) followed by Kolatia Bhendi (52), Iron Bhendi (54), Orka Anamika India (58.66) and Dherosh Chamak (59.33).

viii) Weight/100 seeds (g)

Weight per 100 seeds was also the highest in Shamol Bangla (7.58 g) followed by Orka Anamika India (6.91 g), BARI-1 (6.73 g), Mahira Cross (6.71 g). The lowest per 100 seeds weight was showed in Iron Bhendi (5.80 g). Seed weight was also lower in Orka Anamika Local (5.94 g) and Shomy Hybrid (6.28 g).

ix) YVMV disease incidence (%)

No variety was found to be resistant to YVMV virus. Sobuj Sathi was observed as the highest (47.61%) YVMV infected variety. Virus incidence was also high in Dherosh Chamak (46.66%) followed by Shomy Hybrid (39.16%), Kolatia Bhendi (33.33%) and Mahira Cross (31.38%). Shamol Bangla showed medium tolerance to viral

incidence (22.72%). Orka Anamika India was found as the best among the varieties on the degree of tolerance (4.34%) followed by Iron Bhendi (13.63%) and BARI-1 (26.15%).

x) Yield (g) per plant

It is very much important that the yield per plant depends on the number of fruit (size and shape) and fruit weight. Shamol Bangla was achieved as the highest yielding variety and yield was 520.78 g/plant. High yield was also observed in BARI-1 (425.92 g). The lowest yield was recorded in Kolatia Bhendi (309.26 g). Iron Bhendi (332.46 g), Shomy Hybrid (342.12 g) and Dherosh Chamak (342.68 g) were also observed as the low yield varieties.

Mean yield of Shamol Bangla (SB) variety was maximum compared with the other varieties, and the difference was also statistically highly significant at 5 % level among the ten varieties (Table 3.2)

Total pod yield on the area (t ha^{-1})

Shamol Bangla was recorded the highest total pod yield (17.35 t ha^{-1}). Total pod yield was also found very high in BARI-1 (14.19 t ha^{-1}), Sobuj Sathi (13.11 t ha^{-1}), Mahira Cross (12.96 t ha^{-1}) and Orka Anamika India (12.28 t ha^{-1}). On the other hand, the lowest yield of Kolatia Bhendi pod was 10.30 t ha^{-1}. Iron Bhendi (11.08 t ha^{-1}), Shomy Hybrid (11.40 t ha^{-1}), Dherosh Chamak (11.42 t ha^{-1}) and Orka Anamika Local (11.44 t ha^{-1}) also showed low pod yield.

xi) Biomass (kg) per plant

Total biomass of the plant was measured. Mahira Cross was recorded the highest biomass (2.89 kg/plant) followed by Dherosh Chamak (2.35 kg), Iron Bhendi (2.23 kg), Orka Anamika India (2.19 kg) and Shamol Bangla (2.18 kg). The lowest biomass was counted in Shomy Hybrid (1.89 kg) followed by Sobuj Sathi (1.93 kg).

Table 3.2: Field performance of 10 varieties of okra. Data were recorded after 90 days of seed sowing (Feb-June 2016).

Varieties	**Height (cm) /plant**	**Branches /plant**	**Leaves /plant**	**Flowers /plant**	**Fruits /plant (a)**	**Wt(g)/fruit (c)**	**Seeds /fruit**	**Wt(g) /100 seeds**	**YVMV infected plants (%)**	**Yield (g) /plant (axc)**	**Biomass (kg) /plant**
SB	106.55	3.00	35.33	1.66	25.00	20.83	77.66	7.58	**22.72**	**520.78***	2.18
MC	95.67	3.33	32.66	1.0	21.66	17.95	63.66	6.71	31.38	389.00	2.89
OAL	86.68	3.33	35.66	1.0	20.33	16.88	61	5.94	30.43	343.23	2.06
SH	83.10	3.66	33.00	1.0	20.33	16.82	50.66	6.28	39.16	342.12	1.89
OAI	81.00	3.33	36.66	1.33	21.00	17.55	58.66	6.91	**4.34**	**368.56**	2.19
SS	81.66	3.66	32.33	1.0	21.66	18.16	61.66	6.56	47.61	393.42	1.93
IB	79.59	4.00	32.66	1.33	20.00	16.62	54.00	5.80	**13.63**	**332.46**	2.23
B-1	78.55	3.33	34.00	1.33	22.33	19.07	64.33	6.73	**26.15**	425.92	2.16
KB	91.23	3.66	35.33	1.33	19.33	15.99	52	6.65	33.33	309.26	2.01
DC	83.93	3.66	34.66	1.33	20.33	16.85	59.33	6.54	46.66	342.68	2.35

N.B: **p-value* < 0.001, highly significant difference among the yield of ten varieties.

Table 3.3: Test of significance for yield of varieties under study.

Test /Varieties	SB	MC	OAL	SH	OAI	SS	IB	B1	KB	DC	P-value
1	648.48	437.46	400.4	330.8	476.4	517	400.4	648.48	330.8	364.77	<0.001*
2	476.4	364.77	298.49	330.8	298.49	298.49	298.49	298.49	298.49	364.77	<0.001*
3	437.46	364.77	330.8	364.77	330.8	364.77	298.49	330.8	298.49	298.49	<0.001*
	520.78	389.00	343.23	342.12	368.56	393.42	332.46	425.92	309.26	342.68	<0.001*

* p-value <0.05, significant at 5% level of significance; p-value was calculated using one sample t-test. Mean yield of SB is maximum compared with the other varieties. There is also statistically highly significant difference among the ten varieties at 5 % level of significance.

In this study, total pod yield range values were 10.30 to 17.35 (t ha^{-1}) among the 10 varieties of okra. In fact of Bangladesh, the Shamol Bangla variety of okra was recorded as the highest yielding (520.78 g/plant) variety and the total pod yield was 17.35 (t ha^{-1}) which is within the range value than the other reported values. Aminu *et al*. (2016) reported that the fresh pod yield per plant were okra cultivar Kwadag, Y'ar gagure, Salkade, and Kwadam, in descending order, with yield ranging from 580.38 to 622.67 g. A field experiment of okra was conducted by Ijoyah & Dzer (2012) from June to October during 2010 and 2011, the yield was reported 6.0 and 5.7 (t ha^{-1}). Rashid *et al*. (2002) reported that the highest yield of OK-292 line okra was 18.00 (t ha^{-1}). Ferdous *et al*. (2017) investigated that the highest yield was obtained from the treatment T4 (14.60 t ha^{-1}) and before published this article above Shamol Bangla cultivation season (Feb-June, 2016) as well as record was completed. Saha *et al*. (2016) studied that the highest yield (12.56 t ha^{-1}) was obtained from cultivar Bankim. Nair *et al*. (2017) reported that the highest marketable yield was 21.65 (t ha^{-1}). Benchasri (2011) reported that the highest marketable fruit were recorded on Lucky file 473 at 19.78 and 19.76 (t ha^{-1}) in 2009 and 2010, respectively. Firoz *et al*. (2007) recorded that the highest yield (12.86 t ha^{-1}) was obtained. Chowdhury *et al*. (2014) investigated that the highest yield was harvested from G1OM2 okra (19.62 t ha^{-1}).

3.4 Summary

The main constraint of okra production in Bangladesh is the viral disease caused by Yellow Vein Mosaic Virus (YVMV), which spread by an insect vector namely white fly (*Bemisia tabaci*). I evaluated 10 selected varieties of okra such as SB, MC, OAL, SH, OAI, SS, IB, B1, KB and DC against virus incidence of YVMV under the open field condition for field performance.

Based on the field performance of selected varieties of okra, no variety was found to be resistant against YVMV. SB was found to be the most-high yielding variety (pod yield 17.35 t ha^{-1}) while with respect to viral resistance, OAI was found to be most tolerant variety (virus incidence 4.34%) followed by IB and B1.

CHAPTER FOUR
COMPARATIVE PHYTOCHEMICAL, NUTRITIONAL AND MEDICINAL PROPERTIES OF OKRA

4.1 Introduction

Okra is a vegetable riched in organic and inorganic nutrients that sustain human health and also used partly as feed for animals (Chattopadhyay *et al*., 2011; Ofoefule & Chukwu, 2001; Rahman *et al*., 2012; Wamanda, 2007; Siemonsma & Kouame, 2004; Saifullah & Rabbani, 2009). Different genotypes of okra differ significantly from each other by different characters such as amount of nutrients, phytochemicals, biochemical and medicinal properties. Okra helps to reduce the blood sugar level by regulating the glucose absorbing rate from the intestinal tract (Sabitha *et al*., 2011; Karim *et al*., 2014). Okra is rich in polyphenolic compounds which were confirmed by the identification and quantification from okra seeds and skins (Arapitsas, 2008). From the literature review, it is evident that okra is a nutrient rich vegetable that has high-level of medicinal values for maintaining good health (to control many diseases causing risk of health). All these factors ultimately vary from cultivar to cultivar. A number of okra varieties are under cultivation in Bangladesh. Besides the improved cultural practices, there is a need to grow high yielding nutritent rich cultivars to get more health benefit.

Although okra is nutritionally and medicinally important vegetables but the comparative study on the biochemical, nutritional composition, phytochemical and medicinal values of different varieties of okra cultivated in Bangladesh is not yet widely carried out. This study was carried out to identify and determine the phytochemical and medicinal properties, which are very much importent for food value analysis and assessment of potential for prevention of various diseases. This study included 10 varieties of okra to compare their phytochemical and medicinal properties with a view to identifying the best nutrient rich variety.

4.1.1 Biochemical and Nutritional contents

Different parts of okra are used for its different source of biochemical and nutritional properties (Schippers, 2000; Oomen & Grubben, 1977; Al-Wandawi, 1983; Martin, 1982; Calisir *et al*., 2005; Martin & Rhodes, 1983; Adeboye, 1996). However, the reported main biochemical and nutritional properties are protein, fat, carbohydrate, minerals (calcium, potassium, Fe, Mn, Ni, Zn and other essential minerals), pectins, fibers, caffeine-free substitute, oil, fatty acids (oleic), palmitic acid, lysine, vitamins (vitamin A, *beta-carotene, xanthine,* folates, niacin, vitamin B6-pyridoxine, thiamin and pantothenic acid) of human dietary requirement for maintaining good health.

4.1.2 Phytochemicals, Antioxidants and Medicinal properties

4.1.2.1 Phytochemicals

The word -"Phyto" comes from the Greek word "Phuton" meaning "plants" hence the chemicals found in plants are called phytochemicals. Phenolic compounds are used to protect humans against many deadly diseases such as cancer and heart disease that threaten us. Okra pods containing phytochemicals positively impact on health and cover the benefits of health and many reports have been established that okra is enriched by the phytochemicals (Sunilson *et al*., 2008; Liao *et al*., 2012). For the limited study in our country, the aim of this study was to qualitatively and quantitatively analyze the phytochemicals to detect the phytochemical rich (higher value) variety among the cultivated 10 varieties of okra through various *in vitro* models.

4.1.2.2 Antioxidants

Antioxidants play a considerable function in case of health, as they can control how fast one age through preventing free radicals. In oxidative stress antioxidant may play important role by plant foods or supplement products in various condition (cancer, coronary heart ailment, obesity, type 2 diabetes - T2DM, hypertension and cataract) to maintain the disease free heathy life (Gey, 1990; Monte *et al*., 2014; dailyhealthvalley.com). The antioxidant properties and vitamin C contents of okra also help promote healthy skin and blood. Most of the synthetic antioxidant additives have shown toxic and/or mutagenic outcomes. Consequently attention has been given to naturally obtaining antioxidants. The present study was taken to evaluate the

quantitative analyses to select the antioxidant rich (higher value) variety among the cultivated 10 varieties of okra to maintain various *in vitro* models.

4.1.2.3 Medicinal properties

The arena's attention is now an increasing number of directed towards plant sources for developing antimicrobial and antidiabetic drugs, for the reason that herbal products (natural products) are taken into consideration more secure than synthetic ones. Therefore, such plants have to be investigated to better understand their chemical composition, safety and efficacy (Nascimento *et al*., 2000). There are several published reports mentioning the antimicrobial, antidiabetic and cytotoxic activity of various plant crude extracts (Igoli *et al*., 2005; Alzoreky & Nakahara, 2003; Al-Hatmi *et al*., 2016; Olowa & Nuñeza *et al*., 2013; Vander & Vlietnck, 1991; Aziz *et al*., 1998; El-Gammal & Mansour, 1986; Islam *et al*., 2010; Negi *et al*., 2011; Sankhalkar & Vernekar, 2016; Agbo *et al*., 2008; Alviano & Alviano, 2009; Talib & Mahasneh, 2010; Paterson, 2008; Cheesbrough, 1984; Peirano, 2008; Bhalwar, 2009; Sabitha *et al*., 2012; Karim *et al*., 2014; Uddin *et al*., 2011; Teng, 1993; Harwig & Scott, 1971; McLaughlin *et al*., 1991; Mikolajczak *et al*., 1989; Meyer *et al*., 1982). It is anticipated that there are approximately two and half million species of higher plants and the majority of these have not yet been examined for their pharmacological effects (Ram *et al*., 2004).

Okra is extensively used in ethno medicine in diverse cultures. The different parts of the plant are used widely in conventional traditional medicine such as antidiabetic, antipyretic, diuretic, constipation, expander of blood volume, antispasmodic, dysentery, diarrhea, stomach, bowels, and kidneys catarrhal infections, ardoururinae, dysuria, gonorrhea, cordial, stimulant, demulcent, emollient poultice etc).

Although, many reports have been performed on okra worldwide, the knowledge about the antimicrobial activity of different varieties of okra cultivated in Bangladesh is limited, some of them were executed on the distinguished variety of the plant. However, which okra variety is the best with respect to medicinal properties among the cultivated varieties in our country is still a question. Therefore, comparative study of medicinal properties of 10 varieties of okra will answer the question. The objective of this investigation was to assess the potential antibacterial activity employed *in vitro*

model; *in vitro* antidiabetic activity against alpha amylase, alpha glucosidase inhibition; and cytotoxicity of the pod extract.

4.2 Materials and methods

Materials and methods were divided into two parts- Qualitative (only for phytochemicals study) and Quantitative analysis (for biochemical and nutritional, phytochemical, antioxidant and medicinal study).

4.2.1 Collection of plant materials

Okra seeds from ten selected varieties were collected from different vendors in Bangladesh. Plants were cultivated in the Biochemistry Research Garden, University of Rajshahi, Bangladesh as decribed in Section 3.2.3.1 (Chapter 3) and premature, healthy okra pods were collected.

4.2.2 Methodology

4.2.2.1 Preparation of aqueous and methanolic extract

A total of 800-1200 g okra pod of each variety were weighed, sliced, dried under sunlight and then dried in an oven at temperature bellow 50°C to make suitable for grinding/crushing and weighed again. After grinding, powder was stored in an air-tight polybag in cool, dark and dry place with tag numbers. Powder was extracted with methanol and distilled water separately (1:4 w/v) by maceration for 7 days with occasional stirring. Then it was filtered with moslin cloth and subsequently with filter paper (Whatman No. 1 filter paper). Filtrate was centrifuged at 6000 rpm for 10 min and supernatant was collected. Crude extract was dried to semi solid by air-drying for 10 days at room temperature and stored at 4°C until further use. The aqueous extract was freez-dried to make a semi solid and stored.

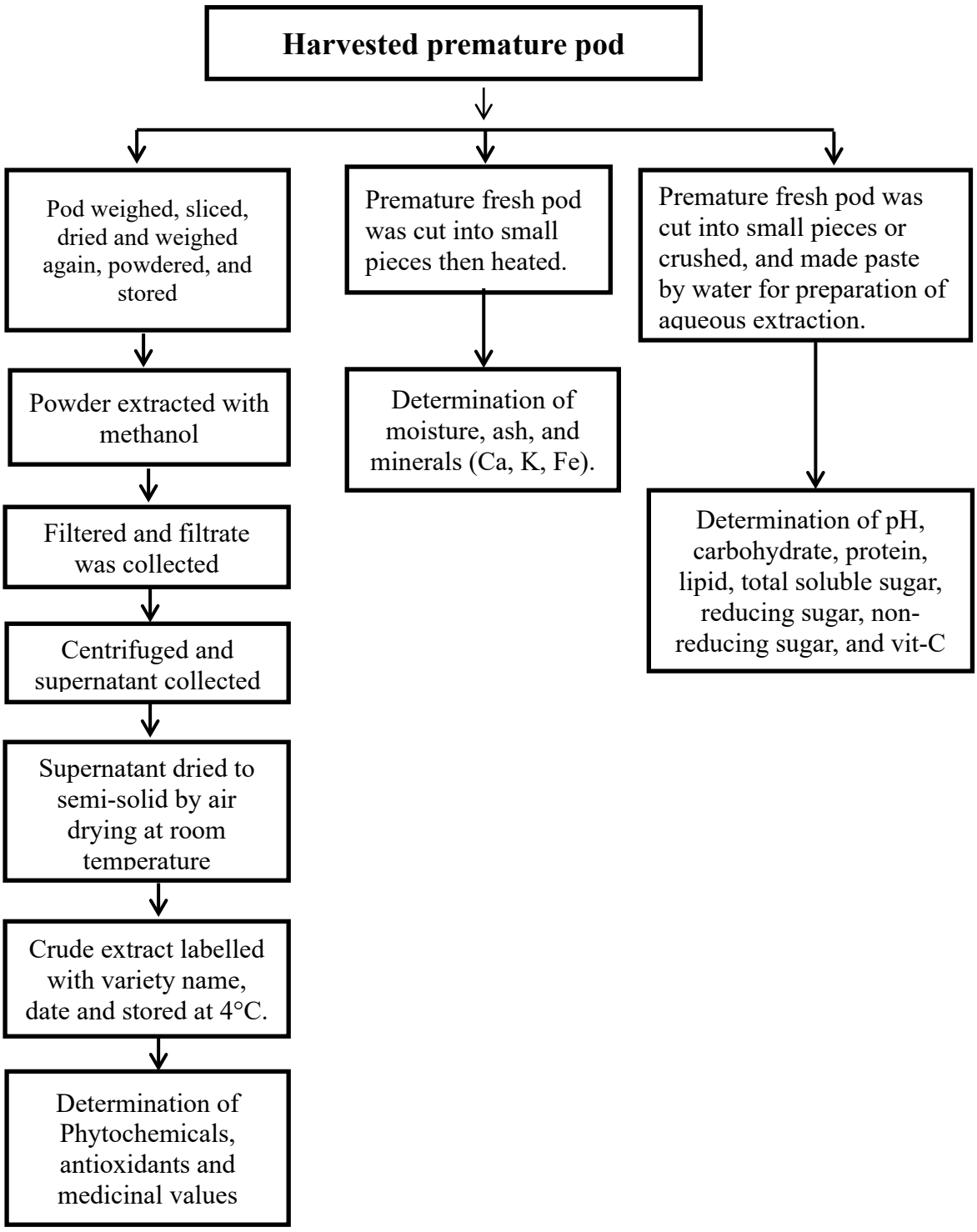

Figure 4.1: Flow chart for the preparation of methanolic and aqueous extracts from premature okra pods.

4.2.3 Qualitative analysis of Phytochemicals

Phytochemicals were qualitatively analysed for the presence of different phytochemicals like alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins by standard methods (Johansen, 1940; Kodangala *et al*., 2010).

4.2.3.1 Alkaloids test

(a) Dragendorff's test

5 ml of distilled water was added to 2 mg of the methanolic extract and 2M Hydrochloric acid was added until an acid reaction occurs. One ml of Dragendorff's reagent was added to this. Formation of orange or orange red precipitate indicates the presence of alkaloids.

(b) Hager's test

A few drops of Hager's reagent were added to two milligrams of the methanolic extract taken in a test tube. Formation of yellow ppt confirms the presence of alkaloids.

c) Wagner's test

A few drops of Wagner's reagent were added to Two milligrams of methanolic extract and acidified with 1.5 %v/v of hydrochloric acid. A yellow or brown ppt. indicates the presence of alkaloids.

(d) Mayer's test

A few drops of the Mayer's reagent were added to two milligrams of methanolic extract. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

4.2.3.2 Carbohydrate test

(a) Anthrone test

Two milligrams of methanolic extract was dissolved in 10 ml of distilled water, filtered and the filtrate was concentrated and 2 ml of anthrone reagent solution was added. Formation of green or blue colour indicates the presence of carbohydrates.

(b) Benedict's test

Two mg of methanolic extract was dissolved in 10 ml of water, filtered and the filtrate was concentrated. To this 5 ml of Benedict's solution was added and boiled for 5 min. Formation of brick red coloured ppt indicates the presence of carbohydrates.

(c) Fehling's test

Two mg of methanolic extract was dissolved in 10 ml of water, filtered and the filtrate was concentrated. To this 1 ml mixture of equal parts of Fehling's solution A and B were added and boiled for a few minutes. Formation of red or brick red coloured precipitate indicates the presence of reducing sugar.

(d) Molisch's test

Two mg of methanolic extract was shaken with 10 ml of water, filtered and the filtrate was concentrated. To this 2 drops of freshly prepared 20% alcoholic solution of α-naphthol was added. 2 ml of conc. sulphuric acid was added so asto form a layer below the mixture. Red violet ring appear, indicating the presence of carbohydrates which disappear on the addition of excess of alkali.

4.2.3.3 Flavonoid test

(a) Shinoda's test

Two milligrams of methanolic extract was dissolved in 5 ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicates the presence of flavonoids.

4.2.3.4 Glycosides

(a) Molisch's test

Two milligrams of methanolic extract was shaken with 10 ml of water, filtered and the filtrate was concentrated. To this 2-3 drops of Molisch's reagent was added, mixed and 2 ml of concentrated sulfuric acid was added carefully through the side of the test tube. Reddish violet ring appear, indicating the presence of glycosides.

4.2.3.5 Triterpenoids

(a) Liebermann - Burchard's test

Two milligrams of methanolic extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a pink colour indicates the presence of triterpenoids.

4.2.3.6 Resins

1 mg of methanolic extract was dissolved in acetone and the solution was poured in distilled water. Turbidity indicates the presence of resins.

4.2.3.7 Saponins

In a test tube containing about 5 ml of a methanolic extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicates the presence of saponins.

4.2.3.8 Steroids

(a) Liebermann-Burchard's test

Two milligrams of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicates the presence of steroids.

(b) Salkowski reaction

Two milligrams of methanolic extract was shaken with chloroform, sulphuric acid was added slowly by the sides of test tube to the chloroform layer. Formation of red colour indicated the presence of steroids.

4.2.3.9 Tannins

A few drops of 5% w/v FeCl3 solution was added to 1-2 ml of the methanolic extract. A green colour indicated the presence of gallotannins, while brown colour indicates the presence of pseudotannins.

4.2.4 Determination of Phytochemicals, Antioxidants and Medicinal properties

4.2.4.1 Determination of Phytochemicals

4.2.4.1.1 Determination of total phenolic content

Total phenolic contents in each extract were determined by the Folin-Ciocalteu reagent (FCR), where Gallic acid was used as a standard (Bakar *et al*., 2009).

a) Principle

The content of total phenolic compounds of different extracts was estimated using FCR. This reagent is used to measure a sample's reducing capacity. The exact mechanism of action of FCR is unknown, but it is believed to contain hetero poly phosphotunstates-molybdates. Sequences of reversible one or two electron reduction reactions lead to formation of blue species, possibly (PMoW11O40)4. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

$$\text{Mo (VI)} + e^{-} \rightarrow \text{Mo (V)}$$

b) Reagents and apparatus

i. Folin-Ciocalteu reagent, FCR (Sigma-Aldrich, Germany)
ii. Sodium carbonate (Sigma-Aldrich, Germany)
iii. Methanol (Sigma-Aldrich, Germany)
iv. Gallic acid (Wako pure chemicals Ltd, Japan)
v. Micropipette (100-1000 µl)
vi. Pipette (1-10 ml)
vii. UV-spectrophotometer (Shimadzu, USA)

c) Procedure

An aliquot of 300 µl of extract was mixed with 2.25 ml of diluted (1:10 in distilled water) FCR and allowed to stand for 5 min at room temperature; and then 2.25 ml of sodium carbonate (60 g/l) solution was added to the mixture. After 90 min incubation at room temperature, the absorbance was measured at 760 nm using a spectrophotometer. Total polyphenolic content (TPC) of the extracts was determined by a modified spectrophotometric method using FCR, calibrated against gallic acid as the reference standard (Singleton & Rossi, 1965; Ough & Amerine, 1988). Quantification of TPC was made by using the calibration curve of gallic acid, which was prepared by diluting stock standard with extraction solvents to yield 50 to 500 mg/L of TPC. The results were calculated according to the calibration curves for gallic acid and the mass fraction of total polyphenols, derived from triplicate analyses and expressed as mg of gallic acid equivalents (GAE) per g of extract dry weight. Values were expressed as means (N=3) ± standard deviations (S.D.)

4.2.4.1.2 Determination of total flavonoids

Total flavonoid contents were estimated using the method described by Bakar *et al*., 2009 and Catechin was used as a standard.

a) Principle

In this method aluminum chloride was used as reagent. Aluminum chloride formed complex with hydroxyl groups of flavonoids present in the samples. Optical density of this complex measured the maximum absorbance at 510 nm.

b) Reagents and apparatus

i) Aluminum chloride (Sigma-Aldrich, Germany)
ii) Methanol (Sigma-Aldrich, Germany)
iii) Catechin (Sigma–Aldrich, USA)
iv) Micropipette (100-1000µl)
v) UV-spectrophotometer (Shimadzu, USA)
vi) Pipette (1-10ml)
vii) Sodium nitrite (Sigma-Aldrich, Germany)
viii) Sodium hydroxide (Sigma-Aldrich, Germany)

c) Procedure

An aliquot of 0.5 ml of the extract was mixed with 2.25 ml of distilled water in a test tube followed by addition of 0.15 ml of 5% $NaNO_2$ solution and kept at room temperature for 6 min. After that, 0.3 ml of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before the addition of 1.0 ml of 1 M NaOH. The mixture was then mixed well using a vortex. The absorbance was measured immediately at 510 nm using a spectrophotometer. Results were calculated and expressed as catechin equivalents per gram of sample dry weight (mg/g).

4.2.4.1.3 Determination of total flavonols

Total flavonols in the plant extracts were evaluated using the method of Kumaran & Karunakaran, 2007.

a) Principle

Total flavonols were estimated by the method of Miliauskas *et al*., 2004 and expressed as mg of qurecitin per gram of extract dry weight. The qurecitin calibration curve was prepared by mixing 2 ml of quercetin solution with 2 ml (20 g/l) of $AlCl_3$ and 6 ml (50 g/l) of sodium acetate. The absorption at 440 nm was read after 2.5 h at 25°C

b) Reagents and apparatus

i. Aluminum chloride (Sigma-Aldrich, Germany)
ii. Sodium acetate (Sigma-Aldrich, Germany)
iii. Quercetin (Sigma–Aldrich, USA)
iv. Micropipette (100-1000µl)
v. UV-spectrophotometer (Shimadzu, USA)
vi. Pipette (1-10ml)

c) Procedure

To 2.0 ml of sample/standard, 2.0 ml of 2% AlCl3 and 3.0 ml (50 g/L) of sodium acetate solutions were added. The absorbance at 440 nm was measured after 2.5 hours at 20°C. Extractives/ standard were evaluated at a final concentration of 0.1 mg/ml. Total content of flavonols was expressed in terms of quercetin equivalent (QU), mg of QU/g of extract drywight.

4.2.4.1.4 Determination of total proanthocyanidins

Determination of content of proanthocyanidins was based on the procedure studied by Sun et al., 1988.

a) Principle

The content of total proanthocyanidins in different extract was determined by the vanillin colorimetric method. In this method, vanillin formed complex with proanthocyanidins present in the samples to give a color complex. This complex has the maximum absorbance at 500 nm.

b) Reagents and apparatus

i) Vanillin (Sigma-Aldrich, Germany)
ii) Catechin (Sigma–Aldrich, USA)
iii) Micropipette (100-1000µl)
iv) Pipette (1-10 ml)
v) UV-spectrophotometer (Shimadzu, USA)

c) Procedure

A volume of 0.5 ml (0.1 mg/ml) of extracts/standard solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Total content of proanthocyanidins was expressed in terms of catechin equivalent, (mg of CA/g of extract dry wt.).

4.2.4.2 Quantitative analysis of Antioxidants

The materials and methods used in this investigation are briefly described below:

1. **Chemicals**

The following chemicals were used in the antioxidative study:

i. Sulphuric acid (E-Mark, Germany)
ii. Ammonium molybdate (E-Merck, Germany)
iii. Methanol (Sigma-Aldrich, Germany)
iv. Ethyl acetate (Sigma-Aldrich, Germany)
v. Dimethyl sulfoxide (DMSO) (E-Merck, Germany)
vi. Potassium ferricyanide [K3Fe(CN)6](E-Merck, Germany)
vii. Sodium phosphate (E-Merck, Germany)
viii. Glacial acetic acid (E-Merck, Germany)
ix. Trichloro acetic acid (TCA) (Loba, India)
x. 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, Germany)
xi. Ascorbic acid (E-Merck, Germany)
xii. Ferric chloride (FeCl3) (E-Merck, Germany)
xiii. 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)
xiv. Potassium persulfate(Sigma-Aldrich, Germany)
xv. p-Nitro-Blue tetrazolium chloride (NBT) (Carl Roth, Germany)
xvi. Hydroxylamine hydrochloride (Sigma-Aldrich, Germany)
xvii. Sulfanilamide (Carl Roth, Germany)
xviii. N-(1-naphthyl) ethylenediamine dihydrochloride (Carl Roth, Germany)
xix. Sodium nitroprusside(E-Merck, Germany)
xx. Sodium carbonate (E-Merck, Germany)
xxi. EDTA (E-Merck, Germany)

2. Equipments

The following equipment was used in the antioxidative study:

i) Spectrophotometer (UV-Visible; ERMA, AE-200, Japan)

4.2.4.2.1 Determination of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was determined by the method of Prieto et al., 2013 with some modifications.

a) Principle

Total antioxidant capacity assay is a spectroscopic method through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH.

$$Mo(VI) + ArOH \longrightarrow Mo(V) + ArO^{\bullet} + H$$

$$Mo(V) + Phosphate \xrightarrow{\text{At acidic } P^H} phosphomolybdenumcomplex$$

Where ArOH = Antioxidant (i.e., phenolic compounds, flavonoids, flavonols and proanthocyanidins)

In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI) and the formation of a green phosphate Mo (V) complex with a maximal absorption at 695 nm.

b) Reagents

i. 0.6 M sulphuric acid
ii. 28 mM sodium phosphate: 0.397 g sodium phosphate was made up to 100 ml with distilled water.
iii. 1% ammonium molybdate: 1 g of ammonium molybdate was dissolved in distilled water a final volume of 100 ml.
iv. Ascorbic Acid (standard: 0.1 mg/ml)

c) Procedure

An aliquot of 0.5 ml of extract at different concentrations was mixed with 3 ml of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate, and incubated at 95°C for 10 min to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank after cooling at room temperature (RT). A standard with Ascorbic Acid at various concentrations was also made in a similar manner for comparison. Increased absorbance of the reaction mixture indicated increase in total antioxidant capacity. All experiments were done in triplicate.

4.2.4.2.2 Determination of Ferric Reducing Antioxidant Power (FRAP)

The reducing power was measured following the method of Oyaizu (1986) with some modification (Oyaizu, 1986; Sun *et al*., 2012).

a) Principle

In ferric reducing antioxidant power (FRAP) assay, there is an increase in absorbance at a pre-specified wavelength as the antioxidant (i.e., phenolic compounds, flavonoids, flavonols and proanthocyanidins) reacts with the lower valency of iron Fe(II) and form charge transfer complexes with the corresponding ligands. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the samples causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Fe^{3+}-ferricyanide + $e^- \longrightarrow$ Fe^{2+}-ferricyanide

$Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO^{\bullet} + H^+$

$Fe(CN)_6^{4-} + Fe^{3+} + K^+ \rightarrow KFe[Fe(CN)_6]$

Where, $Fe[Fe(CN)_6]$:Prussian Blue with λ_{max} = 700 nm. ArOH = Antioxidant (i.e., phenolic compounds, flavonoids, flavonols and proanthocyanidins).

b) Reagents

i. 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution : 1 g of potassium ferricyanide was dissolved in distilled water to a final volume of 100 ml.
ii. 0.2 M potassium buffer
iii. 10% Trichloroacetic acid (TCA) solution: 10 g of trichloro acetic acid was dissolved in distilled water to a final volume of 100 ml.
iv. 0.1% $FeCl_3$ solution: 0.05 g of ferric chloride was dissolved in distilled water to a final volume of 50 ml.
v. Ascorbic acid (standard: 0.1 mg/ml)

c) Procedure

A volume of 250 µL of extract with different concentrations was mixed with 1.75 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min followed by the addition of 1 ml TCA (10%). An aliquot (1 ml) from the incubation mixture was mixed in a test tube with 1 ml of distilled water and 0.2 ml of ferric chloride (0.1%). The absorbance of the resulting solution was measured at 700 nm after 10 minutes. An increased absorbance of the reaction mixture indicates increased reducing power (Farvin *et al*., 2010). A standard with ascorbic acid at various concentrations was also made in a similar manner for comparison.

4.2.4.2.3 Determination of DPPH Free Radical Scavenging Activity

The free radical scavenging activity of various extracts was measured *in vitro* by 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay according to the established method with some modifications (Shirwaikar *et al*., 2006; Kumar *et al*., 2012).

a) Principle

The scavenging of DPPH• by free radical scavenger (i.e., phenolic compounds, flavonoids, flavonols and proanthocyanidins) can be represented as:

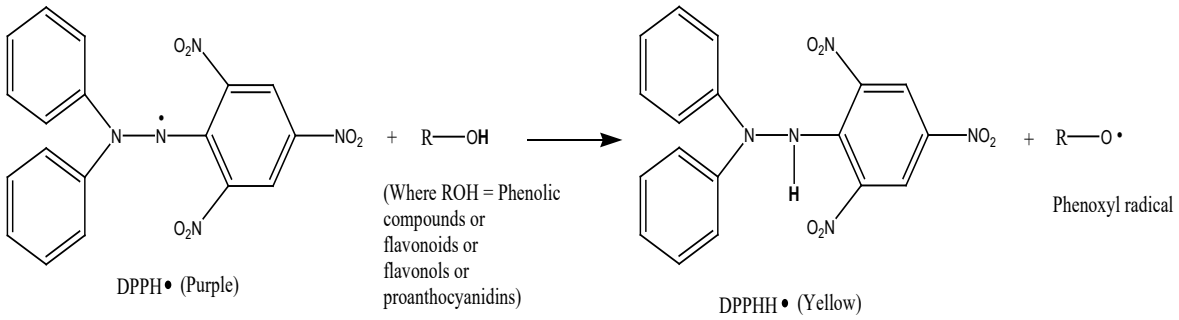

Figure 4.2: DPPH scavenging activity by antioxidants

b) Reagents

i. 0.1 mM DPPH solution in methanol: 0.00394 g of DPPH was dissolved in methanol to a final volume of 100 ml.
ii. Methanol
iii. Ascorbic acid

c) Procedure

Different concentrations of extracts were added at an equal volume (2ml) to a 0.1mM methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Scavenging Activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

Where, $A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample.

Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated. Ascorbic acid was used as positive control.

4.2.4.2.4 Determination of ABTS Radical Scavenging Activity

The antioxidant capacity was estimated in terms of the ABTS●+ radical scavenging activity following the procedure described by (Re *et al*., 1999; Cai *et al*., 2006).

a) Principle

ABTS• assay can be used to determine the antioxidant activity of biological fluids, cells, tissues, natural and other synthetic therapeutical compounds. The ABTS• radical scavenging method is based on the reduction of blue/green ABTS• chromospheres generated from the reaction between ABTS• and potassium persulfate ($K_2S_2O_7$) by an electron-donating antioxidant. The decolourization of the ABTS• chromophoreis measured spectrophotometrically at 734 nm in both lipophilic and hydrophilic medium.

ABTS• (white) + potassium persulfate ($K_2S_2O_7$) →ABTS• (blue/green)

b) Reagents

i. 7 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS•): 19 mg ABTS• for making the volume 50 ml with distilled water.
ii. 2.45 mM potassium persulfate: 3.3 mg $K_2S_2O_7$for 50 ml distilled water.
iii. Ascorbic acid

c) Procedure

ABTS was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12–16 h before use. The ABTS solution (stable for 2 days) was diluted with water to obtain an absorbance at 734 nm of 0.70±0.02. ABTS+ solution (3ml) was added to 1 ml of test sample with various concentrations and mixed vigorously. The absorbance was measured at 734 nm after standing for 6 minutes. Ascorbic acid was used as positive control.

The ABTS radical scavenging activity of the samples was expressed as

$$\% \text{ Scavenging Activity} = [(A_{control} - A_{sample}) / A_{control})] \times 100$$

Where, $A_{control}$ is the absorbance of the blank control (ABTS●+ solution without test sample) and A_{sample} is the absorbance of the test sample.

4.2.4.2.5 Determination of Nitric Oxide (NO) Scavenging Activity

The method of Garrat (1964) was used to determine the nitric oxide radical scavenging activity of plant extracts with some modification (Garratt, 1964).

a) Principle

This assay relies on a diazotization reaction that was originally described by Griess in 1879 which is based on the chemical reaction that uses sulfanilamide or sulfanilic acid and naphthylethylenediamine dihydrochloride (NED) under acidic conditions. Sulfanilamide and NED compete for nitrite in the Griess reaction. Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide.

b) Reagents

i. 10 mM Sodium nitroprusside
ii. Phosphate Buffer Saline (0.02 M), PBS (pH 7.4)
iii. Griess reagent: 1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid)
iv. Naphthylethylenediamine dihydrochloride (NED)

c) Procedure

A volume of 2 ml of 10 mM sodium nitroprusside prepared in 0.5 ml of phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations. The mixture was incubated for 150 min at room temperature. After 150 min incubation, solution was mixed with 0.5 ml of Griess reagent and kept at room temperature for 5 min. After addition of 1 ml of naphthylethylenediamine dichloride (0.1% w/v)] to the mixture it was incubated at room temperature for 30 min, followed by the measurement of absorbance at 546 nm. Ascorbic acid was used as positive control.

The NO radical-scavenging activity of the samples was expressed as

$$\% \text{ Scavenging Activity} = [(A_{control} - A_{sample})/A_{control})] \times 100$$

Where, $A_{control}$ is the absorbance of the blank control (NO radical solution without test sample) and A_{sample} is the absorbance of the test sample.

4.2.4.3 Quantitative analysis of Medicinal properties.

4.2.4.3.1 Cytotoxicity study

This study was carried out using Brine Shrimp Lethality Bioassay (Meyer *et al*., 1982).

1 **Principle of Cytotoxic Effect**

Brine shrimp eggs are hatched in simulated sea-water to get nauplii. Test samples are prepared by the addition of calculated amount of distilled water for obtaining desired concentration of the test sample. The nauplii are counted by visual inspection and are taken in vials containing 10 ml of sea-water. Then samples of different concentrations are added to the pre-marked vials through micropipette. The vials are then left for 24 hours and then the nauplii are counted again to find out the cytotoxicity of the test compound and compared to the results with positive control.

2 Experimental design

2.1 Test materials

a) *Artemia salina*, Leach (brine shrimp eggs)
b) Sea salt (non-ionized, NaCl)
c) Small tank with perforated dividing dam to hatch the shrimp
d) Lamp to attract the nauplii
e) Pipettes (5 ml & 1 ml)
f) Micropipette (10 µl- 100µl adjustable)
g) Glass vials (10 ml).
h) Magnifying glass (3X magnifying glass).

2.2 Preparation of simulated seawater

A 38 g portion of sea-salt (non-ionized NaCl) was weighed accurately and dissolved in one liter of sterilized distilled water and then filtered off to get a clear solution. The pH of the seawater was maintained between 8 and 9 by using $NaHCO_3$ solution.

2.3 Hatching of brine shrimp eggs

Artemia salina, Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 g/L) were added to one side of the tank and this side was covered with paper. The shrimps were allowed for two days (48 h) to hatch and mature as nauplii (larvae). Constant oxygen supply and constant temperature (around 37˚C) was maintained during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through dam. These nauplii were taken for this bioassay.

2.4 Preparation of sample

The test sample (10 mg) of extracts was accurately weighed in a vial and dissolved in 1 ml of pure distilled water to get a concentration of 10 µg/µl. Gallic Acid was also prepared as a positive control in all concentrations. These solutions were used as stock solution.

2.5 Application of the pod extracts and brine shrimp nauplii

Five clean vials were taken for the sample in five concentrations (one vial for each concentration) and another vial was also taken for control. Five ml of sea water was given to each of the vials, and then specific volumes of sample were transferred from the stock solutions to the vials to get the final sample with concentrations of 10, 100, 200, 400 and 800 µg /ml respectively. Gallic Acid was used as a positive control in all concentrations. With the help of a Pasteur pipette 10 living shrimps were taken to each of the sample vials and also to the control. There were three (3) replicates in each concentration. Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 10 ml per test vial.

3 Counting of nauplii

The above mentioned settings were kept for 24 hours (incubation period). The vials were then observed using a magnifying glass and the numbers of survived nauplii in

each vial were counted and noted. The percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} values were determined using probit analysis (Finney, 1952; Busvine, 1971).

4.2.4.3.2 Antibacterial Activity Test

The disc diffusion method (Vander & Vlietnck, 1991) extensively preferable for the preliminary assesment of antimicrobial activity was adopted for the antibacterial activity study.

1 Principle of Antibacterial Effect

The disk diffusion susceptibility method is simple and well-standardized method for determination of the antimicrobial activity of test sample and antibiotics. Bacterial inoculums are applied to the surface of a large agar plate. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 37°C prior to determination of results. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The zones of growth inhibition are measured to the nearest millimeter around each of the antibiotic disks. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Barry, 1976). The diameter of zone of inhibition was proportional to the logarithm of the concentration of the antibiotic.

2 Apparatus and Reagents

i. Filter paper discs (sterilized)
ii. Petri dishes
iii. Nose mask and Hand gloves
iv. Inoculating loop
v. Laminar air flow unit (bio-craft & scientific industries, India)
vi. Sterile cotton
vii. Autoclave (ALP Co. Ltd. KT-30L, Tokyo)
viii. Sterile forceps
ix. Incubator
x. Bunsen burner

xi. Ethanol
xii. Micropipette
xiii. Nutrient Agar Medium (Difco)
xiv. Micropipette (10-100 µ1)
xv. Refrigerator
xvi. Punch machine
xvii. Standard antibiotic (Azithromycin-15 µg/disc) discs

3. Test organisms

Eight pathogenic bacteria were selected for the antibacterial activity test, three of which were gram positive and the remaining were gram negative. The pure cultures were collected from the microbiological research laboratory, Dept. of Biochemistry and Molecular Biology, Rajshahi University. The bacterial strains used for this investigation are listed in the following table:

Table 4.1: List of test bacteria used in antibacterial study.

Gram positive	Gram negative
a) Strepto coccus	*a) Shigella boydii*
b) Bacillus subtilis	*b) Shigella flexneni*
c) Staphylococcus aureus	*c) Salmonella typhi*
-	*d) Escherichia coli*
-	*e) Shigella dysenteriae*

4. Sterilization Procedure

Antimicrobial screening was carried out in a laminar air flow unit and all types of precautions were highly maintained to avoid any contamination during the test. UV light was switched on one hour earlier of the start of the experiment to avoid contamination. Petri dishes and other glassware were sterilized by an autoclave machine at a temperature of 121°C and pressure of 15 lb/sq inch for 30 min. Blank discs were also sterilized and kept in laminar hood under UV light for 30 min.

5. Preparation of the culture media

For demonstrating the antibacterial activity and subculture of the test organisms, nutrient agar media (Difco) was used. For preparation of 100 ml nutrient agar media, 0.5 g peptone, 1 g yeast extract, 0.5 g sodium chloride and 2 g agar were dissolved in distilled water. This composition of the nutrient was maintained throughout the work.

Table 4.2: Composition of nutrient agar medium

Ingredient	Amount
Bacto peptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 g
Bacto agar	2.0 g
Distilled water	100 ml
pH	7.2±0.1 at 25 ^{0}C

6. Preparation of fresh culture

The nutrient agar medium was autoclaved and poured in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized for 30 min. After sterilization, the test tubes were kept in an inclined position (45°) for solidification. These were then incubated at 37°C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. The loop was red heated carefully after each transfer of microorganism to avoid contamination. The inoculated slants were then incubated at 37°C for 24 h to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

7. Preparation of the test plates

The test plates were prepared according to the following procedure:

- **a.** A 15 ml volume of previously prepared nutrient agar medium was poured in each of the clean test tubes and plugged with cotton.
- **b.** The test tubes and a number of petri dishes were sterilized in an autoclave for 30 min and were transferred into laminar air flow unit and then allowed to cool to about 45°C to 50°C.

c. The test organism was transferred from the fresh subculture to the test tube containing 15 ml autoclaved medium with the help of an inoculating loop in an aseptic condition. Then the test tube was gently shaken to get a uniform suspension of the organism.

d. The bacterial suspensions were immediately transferred to the sterile petri dishes in an aseptic area. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms.

e. The media were poured into petri dishes in such a way as to give a uniform depth of approximately 4 mm.

f. Finally, the medium was cooled to room temperature under laminar airflow and was stored in a refrigerator (4°C).

8 Preparation of discs

Three types of discs were prepared for antibacterial screening. These are -

A. Sample discs

Sterilized (BBL, U.S.A) filter paper discs (5 mm in diameter) were prepared with the help of punch machine and were taken in a blank petri dish. Sample solution of desired concentration (10 µl/disc) was applied on the discs with the help of a micropipette in an aseptic condition.

B. Standard discs

These were used to compare the antibacterial activity of test material. In our investigation, Azithromycin (15 µg/discs) was used as standard disc.

C. Solvent control discs

These were prepared using same filter paper (5 mm diameter) and same volume of residual solvent without sample following the same process and condition. These were used as negative control to ensure that the residual solvent and the filter paper themselves was not active.

9. Preparation of test sample

An aliquote of 30, 60 and 90 mg of crude extracts were dissolved in 1 ml methanol in separate glass vial to the concentrations of 30, 60 and 90 µg/µl, respectively for each extract.

10. Placement of the discs and incubation

The following procedure was adopted for the placement of the discs:

a. The dried crude extract discs and standard discs were placed gently on the solidified agar plates seeded with the test organisms with the help of a pair of sterile forceps to ensure contact with the medium.
b. Then the plates were kept in a refrigerator at 4°C for 24 h in order to provide sufficient time to diffuse the antibiotics into the medium.
c. Finally, the plates were incubated at 37°C for 24 h in an incubator.

11 Precaution

The discs were placed in such a way that they were separated from each other about 20 and 15 mm far from the edge of the plate to prevent overlapping the zones of inhibition.

12 Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale.

4.2.4.3.3 *In vitro* Antidiabetic Activity Test

4.2.4.3.3.1 α-Amylase inhibition assay

The inhibition test was carried out according to Miller (Miller, 1959) using (3, 5 dinitrosalicylic acid) DNSA method. A 500 µl volume of extracts at different concentration were added to 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.5 mg/ml of α-amylase solution and were incubated at 37°C for 10 min, followed by addition of 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH-6.9) all the test tubes. The reaction was stopped with 1.0 ml of 3, 5 DNSA reagent. The test tubes were then incubated in

a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples containing various concentrations of extracts prepared .The results were expressed as % inhibition calculated using the formula:

$$\alpha\text{-Amylase inhibition activity} = [1 - \{(A_1-A_2)/A_0\}] \times 100$$

Where A_1 is the absorbance of test sample, A_2 is the absorbance of product control (sample without α-Amylase solution) and A_0 is the absorbance of negative control (α-Amylase without extract).

4.2.4.3.3.2 α-glucosidase inhibition assay

The inhibition test was followed and calculated in the same way as mentioned in subsection 4.2.4.3.3.1. To this analysis, α-glucosidase was used instead of α-amylase.

4.2.5 Determination of moisture, ash and Minerals (Ca, K, Fe)

4.2.5.1 Moisture

Moisture content was determined by the conventional procedure.

Materials:

a) Porcelain crucible
b) Electrical balance
c) Oven
d) Desiccator

Procedure:

About 5 g of each variety of *A. esculentus* premature pods were weighed in a porcelain crucible (which was previously cleaned, heated to 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100°C. It was then cooled in desiccators and weighed again.

Calculation:

Percent of moisture content (g per 100 g of each variety of *A. esculentus* premature pods)

$$= \frac{\text{Weight of ash obtained}}{\text{Weight of the sample}} \times 100$$

4.2.5.2 Ash

Ash content was determined following the method (A.O.A.C, 1980).

Materials:

a) Porcelain crucible
b) Muffle furnace
c) Electrical balance (Mettler H-18)
d) Deseccator

Procedure:

About 5 grams of each variety of *okra* premature pods were weighed in a porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible was placed in a muffle furnace for about four hours at about 600°C. It was then cooled in a desecrator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same and the ash was almost white in color.

Calculation:

Percent of ash content (g per 100 g of each variety of *A. esculentus* premature pods

$$= \frac{\text{Weight of ash obtained}}{\text{Weight of the sample}} \times 100$$

4.2.5.3 Mineral content

Plant samples were prepared by drying and grinding. Then Organic matter was digested and Ca, K and Fe were released by digestion with nitric acid. Ca and Fe were determined by atomic absorption spectrophotometer (Smith & Hieftje, 1983; Sies, 1997).

Reagents

a) Iron accelerator
b) Copper accelerator
c) Concentrated sulphuric acid
d) Catalyst mixture
e) 33% sodium hydroxide
f) 0.05 M sodium hydroxide
g) 0.05 M Hydrochloric acid
h) Methyl red- methyl blue indicator solution
i) 68% Nitric acid
j) 1: 20 diluted HNO_3
k) 1:100 diluted HNO_3
l) 5 M HNO_3
m) $CaCl_2$- solution
n) Acetate buffer solution
o) Azomethine-H reagent
p) Perchloric acid
q) Hydrochloric acid 1:1
r) 0.5 M Barium chloride solution
s) Silver nitrate solution
t) Used two stock solutions and one standered solution of each mineral at different concentration.

Digestion

0.5 g dried plant material was weighed into each of 38 nitrogen digestion tubes. The two remaining tubes were blanks. 5 ml 68 % nitric acid was added to each of all 40 tubes. The content in each tube was mixed and left overnight. The tubes in the digester were placed and covered the tubes with the exhaust manifold. The temperature was set to 125°C. The digester was turn on and continued the digestion for 4 hours after boiling has started. Precaution was taken so that no tubes became dry.

After cooling, the digestion mixture was transferred with distilled water to a 100 ml volumetric flask. The flask was made up to volume with water and mixed and filtered

on a dry filter into a dry bottle, which was closed with a screw cap. The filtrate was kept in the closed bottle. Ca, K and Fe contents in the filtrates were determined.

Determination of minerals

Using a pipette, transfer 20 ml of filtrate was transferred to a 100 ml volumetric flask. The flask was made up to volume with distilled water and was mixed well.

4.2.5.3.1 Estimation of Ca

20 ml diluted filtrate was transferred into a 50 ml volumetric flask and the flask was made up to volume with distilled water and mixed. The content of Ca was measured by atomic absorption spectrometer (AAS). If the reading is higher than the reading of the highest standard solution, then it is need to larger dilution, e.g. 10 ml filtrate into a 50 ml volumetric flask. In this case 1:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 20 ml.

4.2.5.3.2 Estimation of K

10 ml diluted filtrate was transferred into a 50 ml volumetric flask using a pipette. The flask was made up to volume with water and mixed. The content of K was measured by flame photometer. A larger dilution is needed if the reading is higher than the reading of the highest standard solution, e.g. 5 ml volumetric flask. In this case 0:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 10 ml.

4.2.5.3.3 Estimation of Fe

The content of these elements were measured by atomic absorption spectrometer (AAS) directly in the undiluted filtrate.

Calculations

Ca, K,

$$\text{mg per kg plant material} = \frac{a \times 25000}{b \times c}$$

Where

a = mg/l Ca or K measured on atomic absorption spectrometer, flame photometer or spectrophotometer, flame photometer or spectrophotometer,

b = ml diluted filtrate transferred into the 50 ml volumetric flask for determination of Ca or K,

c = Amount of plant material (g) weighted into the digestion tube.

If an additional dilution is made before the transfer to the 50 ml volumetric flask, the result is multiplied by the dilution factor.

$$\text{Fe, mg per kg plant material} = \frac{d \times 100}{c}$$

Where

d = mg/l Fe measured on atomic absorption spectrometer or spectrophotometer,

c = g plant material weighted into the digestion tube.

4.2.6 Quantitative Biochemical and Nutritional analysis

Reconstitution of aqueous extract.

The freeze-dried aqueous extract was reconstituted in distilled deionized water (to make the conc as before) and sed for the determination of biochemical and nutritional properties.

4.2.6.1 Determination of pH

Standard buffer solution:

A standard buffers solutions were prepared by dissolving pH 7.0 and 4.0 buffer tablets (BDH chemicals Ltd. Poole, England) in 100 ml distilled water.

Procedure:

The electrode assembly of the pH meter was dipped into the standard buffer solution of pH 7.0 taken in a clear and dry beaker. The temperature correction knob was set to room temperature (28°C) and the fine adjustment was made by asymmetry potentially knob to pH 7.0. After washing, the electrode assembly was then dipped into a solution of standard pH 4.0 and adjusted to the required pH by fine asymmetry potentially knob. The electrode assembly was raised, washed twice with distilled water, rinsed off with juice of the

Abelmoschus esculentus and then dipped into the juice of the *A. esculentus* premature pods. The pH of the *A. esculentus* premature pods juice was given in the Table 4.11

4.2.6.2 Determination of carbohydrates

The total carbohydrate content was determined by the Phenol suplhuric acid method (Dubois et al., 1956 and Krishnaveni et al., 1984).

Principle

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green colored product with phenol and has absorption maximum at 490 nm.

Materials

- Phenol 5%: Redistilled (reagent grade) phenol (50 g) were dissolved in water and diluted to one liter.
- Sulphuric acid 96% reagent grade.
- Standard Glucose: Stock – 100 mg in 100 ml of water were prepared. Working standard – 10 ml of stock diluted to 100 ml with distilled water.

Procedure

About 100 mg of the sample was weighed into a boiling tube and hydrolysed with 5 ml of 2.5 N-HCl by keeping it in boiling water bath for 3 h and cooled to room temperature. The hydrolysate was neutralised with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuged. Aliquotes of 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard were pipetted out into a series of test tubes. Aliquotes of 0.1 and 0.2 ml of the sample solution were pipetted out in two separate test tubes and the volume in each tube was made up to 1 ml with distilled water. A blank was set with 1 ml of water. Then 1 ml of phenol solution and 5 ml of 96% sulphuric acid was added to each tube and shaken well. After 1 min the contents were placed in a water bath at 25-30°C for 20 min. The absorbance was recorded at 490 nm. The amount of total carbohydrate present in the sample solution was calculated using the standard graph (Appendix 10.1)

Calculation

Absorbance corresponds to 0.1 ml of the test = ‘x’ mg of glucose

% of total carbohydrate present= (‘x’ ÷ 0.1) × 100 mg of glucose

4.2.6.3 Determination of water-soluble protein

Water-soluble proteins content of *okra* premature pods were determined following the method as described by Lowry *et al*. (1951). The extraction was carried out with distilled water.

Reagents:

a) 2% Na_2CO_3 solution in 0.1N NaOH
b) 0.5 copper sulfate in 1% sodium-potassium tartarate.
c) Folin-Ciocalteau reagent (FCR): (Diluted with equal volume of H_2O, just before use).
d) Protein standard: 100 μg/ml. in water.

Procedure:

a) Reagents (a) and (b) were mixed in the ratio 50:1 and diluted reagent (c) just before use.
b) To nine glass test tubes, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 ml of the standard protein solution, respectively were taken and the volume was made up to 1 ml by distilled water. The sample was transferred to a 50 ml volumetric flask and the volume was made upto the mark by distilled water. Water was carefully added avoiding formation of emulsion. 1 ml of the sample was taken in a test tube and a duplicate was made. To each of the tubes 5.0 ml of (a : b) mixture was added and after 10 min, 0.5 ml (FCR) solution was added. Absorbences of the solutions were recorded after 30 min at 650 nm. A standard curve was constructed with the data obtained from the standards and the amount of protein in the sample was calculated from the standard curve (Appendix 10.2).

Calculation:

Percent of protein content (g per 100 g of each variety of *A. esculentus* premature pods)

$$= \frac{\text{Weight of water soluble protein}}{\text{Weight of the sample}} \times 100$$

4.2.6.4 Determination of lipid

Lipid content was determined by the method of Bligh & Dyer (1959).

Reagent:

Mixture of chloroform and ethanol (2: 1 V/V)

Procedure:

About 5 g of dry premature *A. esculentus* pods were first pasted in a mortar with about 10 ml of distilled water. The pasted flesh was transferred to a separating funnel and 30 ml of chloroform-ethanol mixture was added and mixed well. It was then kept overnight at room temperature in the dark. At the end of this period, 20 ml of chloroform and 20 ml of water were further added and mixed. Three layers were seen. A clear lower layer of chloroform containing the entire lipid, a colored aqueous layer of ethanol with all water-soluble materials and a thick pasty inter-phase were seen.

The chloroform layer was carefully collected in a pre-weighed beaker (50 ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of the lipid.

Calculation:

Percent of lipid content (g per 100 g of *A. esculentus* premature pods)

$$= \frac{\text{Weight of lipid obtained}}{\text{Weight of sample taken}} \times 100$$

4.2.6.5 Determination of total soluble sugar

Total sugar content of *A. esculentus* premature pods was determined colorimetrically by the anthrone method as described in Laboratory Manual in Biochemistry (Jayaraman, 1981).

Reagents:

a) **Anthrone reagent:** The anthrone reagent was prepared by dissolving 2 g of anthrone in 1000 ml of concentrated H_2SO_4.

b) **Standard glucose solution:** A standard solution of glucose was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

Extraction of sugar from *A. esculentus* premature pods

Extraction of sugar from *A. esculentus* premature pods was performed following the method described by Loomis & Shull, 1927.

About 5 g of premature pods were separately plunged into boiling ethyl alcohol and allowed to boil for 5-10 min (5 to 10 ml of alcohol was used per g of flesh pulp). The extract was cooled and pasted thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the pasted tissue for three minutes in hot 80 percent alcohol, using 2 to 3 ml of alcohol per g of sample. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatmann No-41 filter paper.

The volume of the extract was evaporated to about one-fourth the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made up to the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made up to the mark with distilled water (working standard).

Procedure:

Aliquot of 1 ml of the pod extract was pipetted into test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on the top of each tube to prevent loss of water by evaporation. The test tubes were heated for 10 min in a boiling water bath and then cooled. A reagent blank was prepared by taking 1 ml of water and 4 of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter.

A standard curve of glucose was prepared by taking 0.0, 0.1 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 10, 20, 40, 60, 80 and 100 μg of glucose respectively and made the volume up to 1 ml with distilled

water. Then 4 ml of anthrone reagent was added to each test tube and mixed well. The absorbance was measured at 680 nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

The amount of total sugar was calculated from the standard curve of glucose (Appendix 10.3). Finally, the percentage of total sugar present in the *A. esculentus* premature pods was determined using the formula given below. The standard curve of glucose is given in appendix no. (10.3).

Calculation:

Percent of total sugar (g per 100 g of *A. esculentus* premature pods)

$$= \frac{\text{Weight of sugar obtained}}{\text{Weight of sample}} \times 100$$

4.2.6.6 Determination of reducing sugar

Reducing sugar content of the *A. esculentus* premature pods was determined by dinitrosalicylic acid method (Miller, 1959).

Reagents:

a) Dinitrosalicylic acid (DNS) reagent. Simultaneously 1 g of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulfite were placed in a beaker and mixed with 100 ml of 1% NaOH solution by stirring.

b) 40% solution of Rochelle salt.

Extraction of sugar from premature pods

Sugar was extracted from *A. esculentus* premature pods by the method as described in section (4.2.6.5).

Procedure:

Aliquot of 3 ml of the extract was pipette into test tubes and 3 ml of DNS reagent added to each of the solutions and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After developing the color, 1 ml of 40% Rochelle salt was added while the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3 ml of

water and 3 ml of DNS reagent in a tube and treated similarly. The absorbances of the solutions were taken at 575 nm in a colorimeter. The amount of reducing sugar was calculated from the standard curve of glucose (Appendix 10.4).

Calculation:

The percent of reducing sugar (g per 100 g of *A. esculentus* premature pods)

$$= \frac{\text{Weight of reducing sugar obtained}}{\text{Weight of the ssample}} \times 100$$

4.2.6.7 Determination of sucrose

Sucrose content was determined by the following formula (Rangama, 1979). Percent of Sucrose or non-reducing sugar = (% Total sugar - % Reducing sugar) × 0.95

4.2.6.8 Determination of vitamin C

Vitamin C content of sample was determined by the titramatric Method (Bessey & King, 1933).

Principle:

Ascorbic acid (Vitamin-C) as the name implies possess the usual acidic properties (Donation of H^+ ion). The acidity of Vitamin C is not due to the carboxylic group tied up in loctone form but is due to the ionization of enol group. The method of the estimation of Vitamin C is based on the stoicheometric reduction of the dye 2, 6-dichlorophenel endophenol to colorless compound by ascorbic acid. The titration is conducted in the presence of metaphosphoric acid, in order to inhibit the oxidation of ascorbic acid catalyzed by certain metallic ion (such as cupric and silver ion present in distilled water). In aqueous system this vitamin is easily oxidized, the stability increases with the increase in pH. Metaphosphoric acid stabilizes the solution by lowering the pH. As a result of titration, vitamin-C is oxidized to dehydro ascorbic acid.

Reagents:

a) Dye Solution: 200 mg of 2, 6 dechlorophenol endophenol and 210 mg of sodium bicarbonate were dissolved in distilled water and made up to 100 ml. The solution was filtered.

b) 3% Metaphosphoric acid reagent: 3 g of metaphosphoric acid was dissolved in 80 ml of acetic acid and made up to 100 ml with distilled water.

c) Standard Vitamin-C Solution (0.1 mg/ml): 10mg of pure vitamin-C was dissolved in 3% metaphosphoric acid and made up to 100 ml with 3% Metaphosphoric acid.

Procedure:

10 ml of standard vitamin-C (Ascorbic acid) solution was taken in a conical flask and titrate it with the dye solution. About five grams of sample were cut into small pieces and homogenized well with 3% metaphosphoric acid (approximately 30ml) and filtered it through double layer of muslin cloth. The filtrate was centrifuged at 3000 r.p.m. for 10 minutes and clear supernatant was titrated against 2, 6-dechlorophenol-endophenol solutions. The amount of vitamin-C present in the extract was determined by comparing with the titration result of standard vitamin-C solution.

Calculation:

Vitamin-C content of the sample was calculated by the following equation:

10 ml Standard Vitamin-C Solution = 1 mg of vitamin C per 100 g sample

$$= \frac{\frac{Y \times W}{X}}{} \times 100$$

Here, X= Volume of sample solution required to titrate the dye solution

Y= Volume of standard Vit-C solution required to titrate the dye solution.

W= Weight of sample taken

4.3 Results and Discussion

4.3.1 Qualitative analysis of phytochemicals

The phytochemical constituents are accountable for therapeutic potential of plant species. In this research the qualitative phytochemical analysis of 10 okra varieties premature pods was achieved and the results are shown in Table 4.3.

Table 4.3: The qualitative phytochemical profile of methanolic extract of 10 varieties of *okra*

Name of the test	Result of-the qualitative test for Methanolic extract									
	SB	MC	OAL	SH	OAI	SS	IB	B1	KB	DC
Alkaloids:										
Dragendorff's test	+	-	-	+	+	-	-	-	+	-
Hager's test	+	+	-	-	+	+	-	+	-	+
Wagner's test	Br+	-	Br+	Br+	-	-	Br+	-	Br+	-
Mayer's test	+	-	+	+	-	-	+	-	+	-
Carbohydrates:										
Anthrone test	+	-	-	+	+	-	-	-	+	+
Benedict's test	+	+	+	-	-	+	+	+	-	-
Fehling's test	+	-	-	+	+	-	-	-	+	+
Molisch's test	+	+	-	-	-	+	-	+	-	-
Flavonoids:										
Shinoda's test	+	+	+	+	+	+	+	+	+	+
Glycosides:										
Molisch's test	+	+	+	+	+	+	+	+	+	+
Triterpenoids:										
Liebermann-Burchard	-	-	+	-	-	-	+	-	-	-
Resins:	+	+	+	+	+	+	+	+	+	+
Saponins:	+	+	+	+	+	+	+	+	+	+
Steroids:										
Liebermann-Burchard	Gr+	-	-	Gr+	Gr+	-	-	-	Gr+	Gr+
Salkowski reaction	-	-	-	-	-	-	-	-	-	-
Tannins:	Gr+	Gr+	Br+	Br+	Gr+	Gr+	Br+	Gr+	Br+	Gr+

Note: "+" indicates the presence of the relevant phytoconstituents, whereas "–" indicates the absence, Br=Brown, Gr=Green.

Qualitative phytochemical analysis of crude methanolic extracts of 10 varieties of okra pods showed the presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins. So, this plant might be right choice to serve the therapeutic purposes. Though the information is limited for okra in Bangladesh, qualitative phytochemical analysis of *Abelmoschus esculentus* has found positive result which was published by some reporters. Abobaker *et al*. (2017) reported that the qualitative and quantitive phytochemical analysis of fruits and leaves of aqueous and ethanolic crude extracts of this plant showed the presence of various phytochemical constituents such as tannins, steroid, flavonoids, saponin, alkaloids, anthraquinones, phenol, terpenoids and cardiac glycosides, while resin is present in the leaves aqueous crude extracts and absent in ethanolic crude extracts of fruits. They also mentioned that the chemical components have a potential for the treatment of various diseases. Shah & Yadav (2015) reported that qualitative phytochemical analysis of the leaf extract of *Sarcochlamys pulcherrima* revealed the presence most of the biochemicals tested for such as carbohydrate, protein, alkaloid, tannin, flavanoid, steroids, terpenoids, phenol, saponin and glycoside. They determined that the presence of various phytochemicals in the tested plant reveals that this plant may be a good source for production of new drugs for various ailments. Jain *et al*. (2011) reported that the ethanolic and aqueous extract of okra fruits were subjected to phytochemical investigation which showed the presence of carbohydrate, gums and mucilages, proteins, phytosterols, flavonoids, tannins, phenolic compounds and volatile oil. Alam *et al*. (2017) reported that the phytochemical analysis of the methanolic extract of *Abelmoschus esculentus* seeds showed the presence of Alkaloid, Flavonoids and Tannin types of compounds.

Shankul Kumar (2014) reported that the phytochemical screening of okra showed the presence of Carbohydrate, Protein and Amino acid, flavonoids, phytosterol, and volatile oil. Islam *et al*. (2016) reported that the qualitative test identified the presence of alkaloids, flavonoids, flavonols, resins, saponins, proanthocyanidins, coumarins, steroids and tannins in the fruit extracts of *Ficus recemosa*.

4.3.2 Phytochemical, Antioxidants and Medicinal properties

4.3.2.1 Quantitative analysis of phytochemicals

4.3.2.1.1 Total Phenolics

All of the 10 okra variety extracts were standardized for their phenolic content. The calibration curve showed linearity for Gallic acid (GA) in the range of 5-120 µg/ml, with a correlation co-efficient (R^2) of 0.998 and equation **y** =0.0051x-0.0107, where y is the absorbance at 760nm and x is the concentration of Gallic acid in µg/ml (Appendix 10.5). Total phenolic contents (TPC) were calculated using the formula as stated below.

$$TPC = (C \times V) \div m$$

Where TPC was expressed in mg/g of the dry extracts as Gallic acid equivalent (GAE), "C" is the concentration of GA, obtained from the calibration curve, "V" is the volume of plant extract in ml and "m" is the dry weight of plant in g. Again the concentration of Gallic acid (C) in mg/ml was calculated from the straight line equation of the standard colorimetric curve using the following formula,

C = [Absorbance of sample(y) − distance of the straight line from the origin of the graph] ÷ [slop of the equation ×1000]

In this study, SB demonstrated the highest amount of phenolics (11.37±0.39 mg of GAE/g of extract dw) compared to other 09 varieties (Table 4.6 and Figure 4.3).

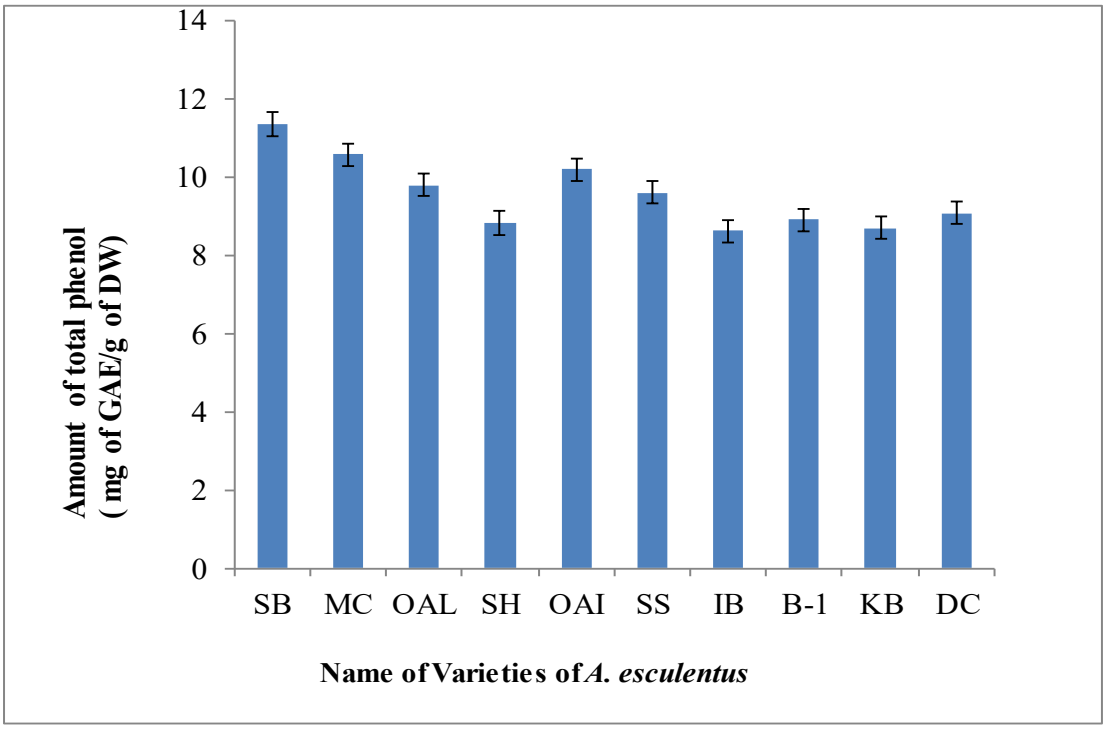

Figure 4.3: Total phenolic content of different varieties extracts of *A. esculentus*. Result are expressed as mean±SEM (n=3), GAE=Gallic Acid Equivalent.

In this study, SB premature pod extract contained the highest amount of phenolics 11.37±0.39 mg of GAE/g of extract dry weight and total phenolic range values were 8.64±0.64 to 11.37±0.39 mg of GAE/g of extract dry weight among the 10 varieties of okra. Above value is within the range and mostly consistent than the other reported values. Shah & Yadav (2015) reported that the total phenolic content of the methanolic leaf extract is found to be 29.42±0.006 mg/g. Ayushi *et al*. (2016) reported that the total phenolics present in the *Abelmoschus esculentus* leaf extract were 9.61 mg of Gallic Acid Equivalent (GAE)/g. According to Khomsug *et al*. (2010), total phenolic content of pulped and seeds of okra extracts as 10.75±0.02mg GAE/100g extract and 142.48±0.02mg GAE/100g extract. Nwachukwu *et al*. (2014) reported that the total highest phenolics content was found in young leaves (0.99 mg TNE/1 g). Ahiakpa *et al*. (2014) reported that the Kortebortor ASR registered was showed the highest total phenolic content of (63.22±3.95 µg/g/GAE) in the aqueous extract of okra. Reports of Weng *et al*. (2005) and USDA (1998) were mentioned that the total phenolic composition of the accessions of okra compares well with common fruits and vegetables noted for their relatively high phenolic constituents such as cranberries

(52.72±2.15 mg/g), apple (29.63±0.64 mg/g), strawberries (16.00±0.12 mg/g), pineapple (9.43±0.15 mg/g), banana (9.04±0.32 mg/g), lemon (8.19±0.35 mg/g), orange (8.12±0.11 mg/g), pear (7.06±0.16 mg/g), and grape (4.96±0.26 mg/g). Sindhu & Puri (2016) reported that the contents of total polyphenols and total polysaccharides were 29.5% and 14.8% in okra seeds and 1.25% and 43.1% in okra skin, respectively. Caluete *et al*. (2015) studied that the extract of leaves showed the content of phenolic compounds of 19.27 mg of GA/g. Islam *et al*. (2016) reported that the quantitative test determined the highest content of phenolics were 56.90±1.37 mg gallic acid/ g dry weight in the ethanolic extract of fruit of *Ficus recemosa.* Mamun *et al*. (2016) investigated that out of the five extracts, the acetone extract of local variety of *Allium sativum* possessed the highest content of phenolics were 110.76±1.9 mg of gallic acid equivalent/g of dry extract.

4.3.2.1.2 Total Flavonoids Content

All the 10 varieties extracts of okra were standardized for their content of total flavonoids. The calibration curve showed linearity for Catechin (CA) in the range of 5-150 µg/ml, with a correlation co-efficient (R^2) of 0.9996 and equation **y** =0.0043x-0.0087, where y is the absorbance at 510 nm and x is the concentration of Catechin in µg/ml (Appendix 10.6). Total flavonoids content of 10 varieties extracts of *Abelmoschus esculentus* were calculated using the following formula,

$$TFC = (C \times V) \div m$$

Where, TFC is the total flavonoids content in mg/g of the dry extracts as Catechin equivalent (CAE), "C" is the concentration of Catechin, established from the calibration curve in mg/ml, "V" is the volume of plant extract in ml and "m" is the weight of dry plant extract in g. Again the concentration of Catechin in mg/ml was calculated from the straight line equation of the standard colorimetric curve using the following formula,

C = [Absorbance of sample(y) − distance of the straight line from the origin of the graph] ÷ [slop of the equation ×1000]

From this study, it was observed that the total flavonoid ranged from 6.70±0.88 to 9.88±0.09 mg of CAT/g of extract dw) among the varieties under study where SB contained the highest amount (Table 4.6 and Figure 4.4).

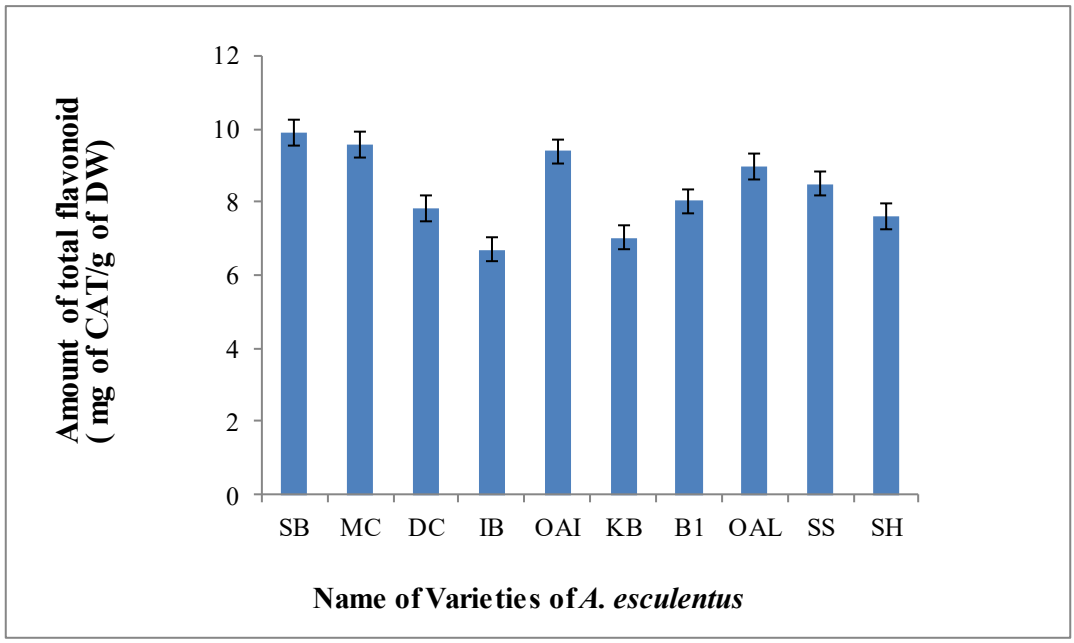

Figure 4.4: Total flavonoid content of different varieties extracts of *A. esculentus*. Result are expressed as mean±SEM (n=3), CAT=Catechin.

The results were consistent with other reported values. Nwachukwu *et al*. (2014) reported that the highest total flavonoid in mature okra leaves was 0.79 mgQE/g. Ahiakpa *et al*. (2014) investigated that the Agric short fruit ethanol extract of okra showed the highest amount of total flavonoid 5159.21±12.90 µg/g/QE. Adelakun *et al*. (2009) studied on okra seed flour in Nigeria and Thailand who obtained 32.54±32.42 mg/g flavonidsfor blanched okra seeds, 48.3±0.00 mg/g for raw okra seeds, 51.28 mg/g for soaked okra seeds. For both cases they concluded that okra contains small amount of flavonoid. Ayushi *et al*. (2016) reported that the total flavonoids present in the *Abelmoschus esculentus* leaf extract were 9.25 mg QE) /g. Shah & Yadav (2015) reported that the total flavonoid contents of the leaf extract of *Sarcochlamys pulcherrima* was found to be 1.091± 0.008 mg/g. Islam *et al*. (2016) reported that the quantitative test determined the highest content of flavonoids at 32.33±2.60 mg of catechin/g dry weight in the ethanolic extract of fruit of *Ficus recemosa.* Mamun *et al*. (2016) reported that out of the five extracts, the acetone

extract of local variety of *Allium sativum* possessed the highest content of flavonoids at 43.32±2.7 mg of catechin equivalent/g of dry extract.

4.3.2.1.3 Total Flavonols Content

Total flavonols content was confirmed using the calibration curve of Quercetin The curve showed linearity for (QU) in the range of 0.5-3.0 µg/ml, with a correlation co-efficient (R^2) of 0.9993 and equation **y** =0.2245x+0.0106, where y is the absorbance at 440 nm and x is the concentration of Quercetin in µg/ml (Appendix 10.7). Total flavonols content was calculated using the following formula,

$$TFC = (C \times V) \div m$$

Where, TFC is the total flavonols content in mg/g of the dry extracts as Quercetin equivalent (QUE), "C" is the concentration of Quercetin, established from the calibration curve in mg/ml, "V" is the volume of plant extract in ml and "m" is the weight of dry plant extract in g. Again the concentration of Quercetin in mg/ml was calculated from the straight line equation of the standard colorimetric curve using the following formula,

C = [Absorbance of sample(y) − distance of the straight line from the origin of the graph] ÷ [slop of the equation ×1000]

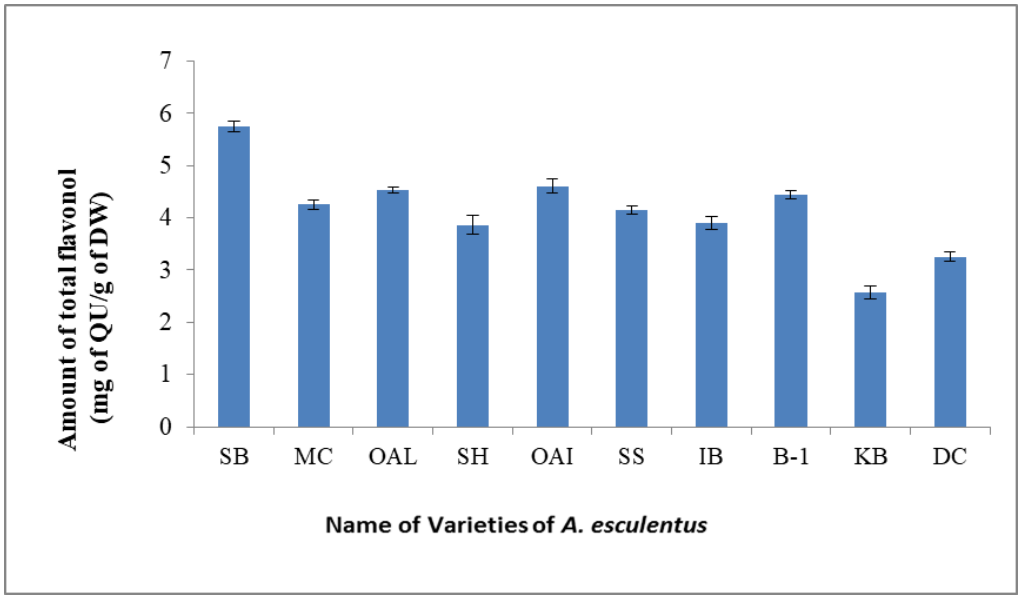

Figure 4.5: Total flavonols content of different varieties extracts of *Abelmoschus esculentus*. Result are expressed as mean±SEM (n=3), QU=Quercetin.

In this study, total flavonol ranged from 2.57±0.11 to 5.75±0.09 mg of QU/g of extract dry weight among the varieties under study where SB pod extract contained the highest amount (Table 4.6 and Figure 4.5). The values are within the range of other reported values. Ayushi *et al*. (2016) reported the total flavonols in okra leaf extract to be 6.12 mg (QE/g) dw. Arapitsas (2008) reported that the okra seed is mainly constituted of oligomeric catechins (2.5 mg/g of seeds) and flavonol derivatives (3.4 mg/g of seeds). Islam *et al*. (2016) reported the highest content of flavonols in the ethanolic extract of *Ficus recemosa* fruit to be 4.21±.19 mg of QE/ g dry weight. Mamun *et al*. (2016) reported that out of the five extracts, the acetone extract of local variety of *Allium sativum* possessed the highest content of flavonols at 15.31±2.3 mg of quercetin equivalent/g of dry extract.

4.3.2.1.4 Total Proanthocyanidins Content

Total proanthocyanidin was calculated using the calibration curve of Catechin that showed linearity in the range of 5-200 µg/ml, with a correlation co-efficient (R^2) of 0.9977 and equation **y** = 0.0096x-0.0107, where y is the absorbance at 500 nm and x is the concentration of Catechin in µg/ml (Appendix 10.8). The formula used was as follows:

$$TPC = (C \times V) \div m$$

Where, TPC is the total proanthocyanidins content in mg/g of the dry extracts as Catechin equivalent (CAE), "C" is the concentration of Catechin, established from the calibration curve in mg/ml, "V" is the volume of plant extract in ml and "m" is the weight of dry plant extract in g. Again the concentration of Catechin in mg/ml was calculated from the straight line equation of the standard colorimetric curve using the following formula,

C = [Absorbance of sample(y) − distance of the straight line from the origin of the graph] ÷ [slop of the equation ×1000]

Orka Anamika India (OAI) contained the highest amount of proanthocyanidin 7.25±0.27 mg of CAT/g of extract dry weight among the 10 varieties extract and the proanthocyanidin content of other 09 varieties extracts were 6.86±0.52, 5.92±0.47, 5.60±0.42, 5.11±0.58, 5.84±0.24, 5.48±0.36, 6.09±0.14, 4.57±0.11 and 6.65±0.59 mg of CAT/g of dry extract for SB, MC, OAL, SH, SS, IB, B-1, KB and DC respectively

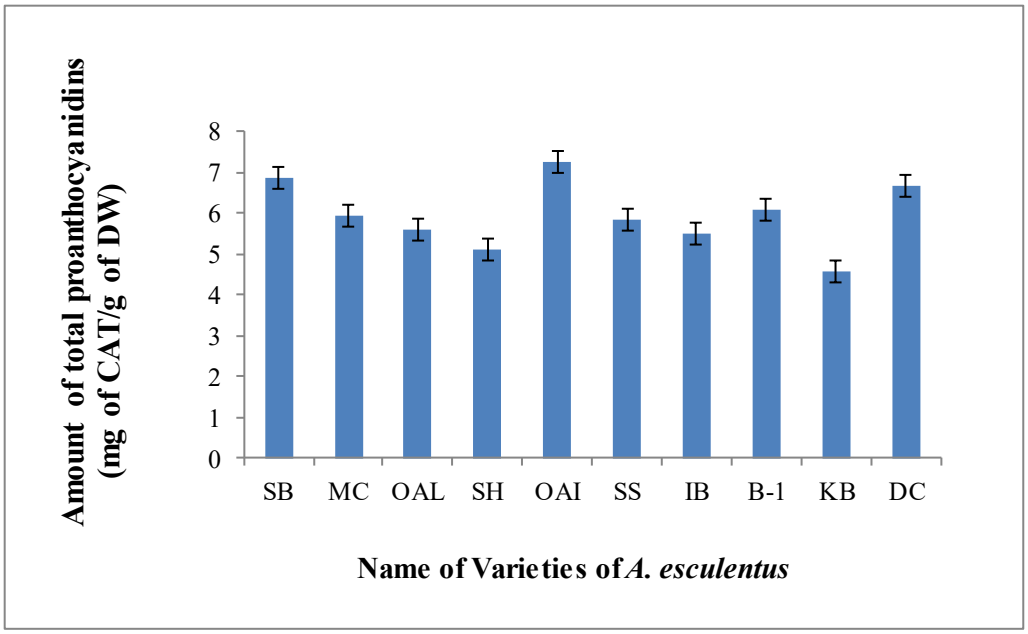

Figure 4.6: Total proanthocyanidins content of different varieties of *Abelmoschus esculentus*. Result are expressed as mean±SEM (n=3), CAT=Catechin.

In this study, total proanthocyanidin ranged from 4.57±0.11 to 7.25±0.27 mg CAT/g of extract dry weight among the varieties under study (Table 4.6 and Figure 4.6), of them OAI contained the highest amount. The values were consistent with the other reported values. Khomsug *et al*. (2010) found procycanidin B2 (mean value was 675.20±0.01 mg/100g) as predominant phenolic compound followed by procycanidin B1 (mean value was 289.40±0.03 mg/100g and rutin in seeds of okra. In pulped seed catechin, procyanidin B2 (mean value was 26.50±0.01 mg/100g), epicatechin and rutin are reported to be present. Islam *et al*. (2016) reported that the quantitative test determined the highest content of proanthocyanidins at 12.59±.39 mg catechin/ g dry weight in the ethanolic extract of fruit of *Ficus recemosa.* Mamun *et al*. (2016) reported that out of the five extracts, the acetone extract of local variety of *Allium sativum* possessed the highest content of proanthocyanidins at 8.54±0.5 mg of catechin equivalent/g of dry extract.

4.3.2.2 Antioxidants

4.3.2.2.1 Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of extracts from 10 okra varieties are shown in Figure 4.7. The total antioxidant potentials was calculated from their ability to reduce Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The extracts demonstrated lower antioxidant activity compared to standard vitamin C at all the concentrations. The total antioxidant activity of 10 varieties under study was found to increase with the increasing concentration of the extracts. The antioxidant capacity of 10 varieties extracts of *Abelmoschus esculentus* may be attributed to their chemical composition and phenolic content. SB extract showed the highest antioxidant capacity at the 160 µg/ml concentration whereas B1 extract attribute the lowest antioxidant potentiality. The total antioxidant capacity of 10 different varieties extracts were in the order SB > OAI > IB > OAL >MC > SS > KB > DC > SH > B1 (Table 4.6).

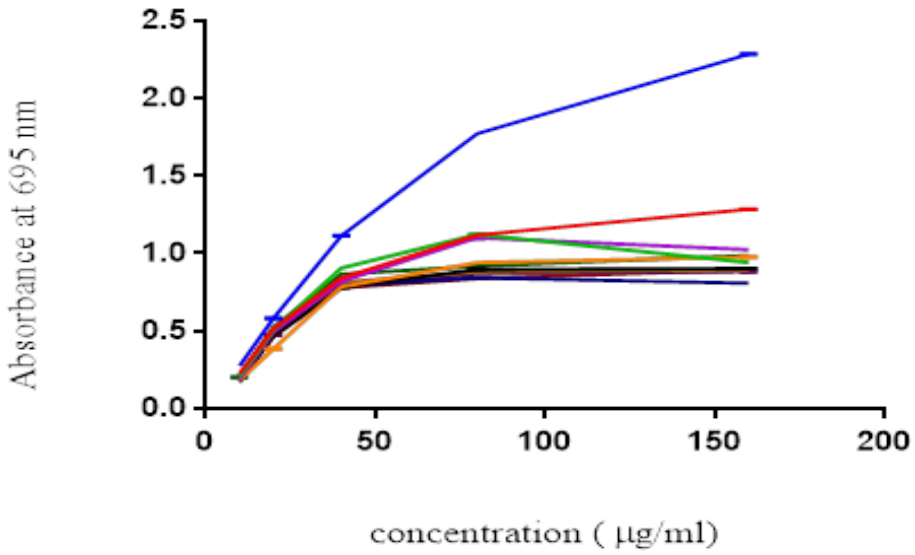

Figure 4.7: Total antioxidant capacity of 10 varieties extracts of *A. esculentus* and Standard. (—) AA, (—) SB, (—) MC, (—) OAI, (—) OAL, (—) SS, (—) DC, (—) B1, (—) SH, (—) KB, (—) IB.

The total antioxidant activity of 10 varieties extracts of *A. esculentus* was found to increase with the increasing concentration of the extracts. Jayaprakasha *et al*. (2008)

investigated that the total antioxidant activity of citrus fruits was due to the existence of phenolics, flavonoids and ascorbic acid. The antioxidant capacity may be attributed to their chemical composition and phenolic content. The total antioxidant capacity of 10 different varieties ranged from 0.809±0.055 to 1.285±0.013 at 160 µg/ml concentration. SB extract showed the highest antioxidant capacity, whereas B1 attributed the lowest potentiality. Mamun *et al*. (2016) studied the acetone extract of local variety of *Allium sativum.*to demonstrate total antioxidant capacity of 0.35 at 100 µg/ml.

4.3.2.2.2 Ferric Reducing Antioxidant Power assay

In ferric reducing antioxidant assay, the presence of reductant in the antioxidant sample causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+}/ferrous form, so the reducing power of the sample can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The Ferrous reducing antioxidant capacity of *Abelmoschus esculentus* is shown in Figure 4.8. The sample extracts showed lower reducing activity than standard ascorbic acid. The reducing activity increased with the increasing concentration of the sample. FRAP is an extensively utilized method for antioxidant determination and has been used for the assessment of the antioxidant and reducing power of plant sample. The FRAP assay gives a direct estimation of the antioxidants or reductants present in a sample based on its ability to reduce the Fe^{3+}/Fe^{2+} couple. The ferric reducing antioxidant capacity of different extract of 10 varieties extracts of *Abelmoschus esculentus* followed the order SB > IB > MC > OAI > OAL > SS > KB > DC > SH > B1 (Table 4.6). Figure 4.8 shows that SB extract at 120 µg/ml possessed the highest reducing potential compared to other extracts.

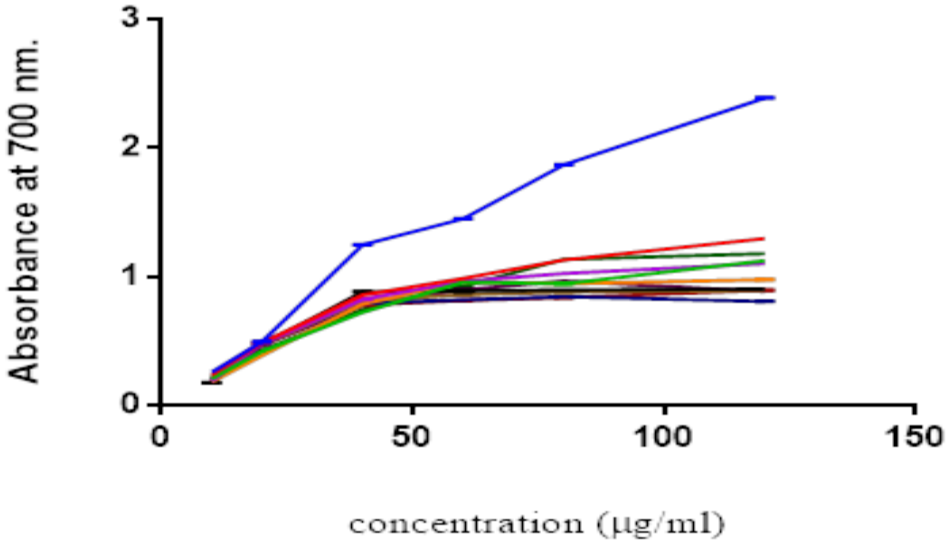

Figure 4.8: Ferric reducing antioxidant capacity of 10 varieties extracts of *Abelmoschus esculentus* and Standard. (—) AA, (—) SB, (—) MC, (—) OAI, (—) OAL, (—) SS, (—) DC, (—) B1, (—) SH, (—) KB, (—) IB.

In ferric reducing antioxidant assay, 10 varieties extracts of *A. esculentus* was observed to increase with the increasing concentration of the extracts. The sample extracts showed lower reducing activity than standard ascorbic acid. FRAP assay, SB extract showed the highest antioxidant capacity (1.293±0.019) at the 120 µg/ml concentration whereas B1 extract attribute lowest antioxidant potentiality. The ferric reducing antioxidant capacity of 10 different varieties premature pod extracts were in the range of 0.801±0.169 to 1.293±0.019 (120 µg/ml). Mamun *et al*. (2016) studied that the ferric reducing antioxidant power assay acetone extract of local variety of *Allium sativum* showed the highest activity were 0.75 (100µg/ml). Ayushi *et al*. (2016) reported that the reducing power of the *A. esculentus* was found to be remarkably greater than that of the standard (Ascorbic acid), at the highest evaluated concentration of 800 µg/ml, confirming thereby the antioxidant potential of *A. esculentus* leaves.

4.3.2.2.3 DPPH Radical Scavenging Activity

In the present investigation, the different concentration of sample and standard exhibited antioxidant activities in a dose dependent manner on the DPPH radical scavenging assay. The scavenging activity of 10 varieties extracts of *Abelmoschus esculentus* was moderate when compared to standard vitamin C. DPPH scavenging ability may depend on the amount of total flavonoids and total phenolics in the extracts. Figure 4.9 showed free radical scavenging activity of the 10 varieties extracts of *Abelmoschus esculentus*. At a concentration of 300 µg/ml, the scavenging activity of SB, MC, OAI, OAL, SS, DC, B1, SH, KB and IB were 76.00%, 72.66%, 71.00%, 66.33%, 65.66%, 65.00%, 61.00%, 60.33%, 60.00% and 55.66% respectively, while at the same concentration, the activity of AA was 86.33%. Thus SB, MC, OAI, OAL, SS, DC, B1, SH, KB and IB exhibited moderate free radical scavenging activity. The IC_{50} of SB, MC, OAI, OAL, SS, DC, B1, SH, KB and IB were higher compared with the IC_{50} value of AA. The inhibitory activity of different extractives was in the order of SB>MC>OAI>OAL >SS>DC>B1>SH>KB>IB (Table 4.6).

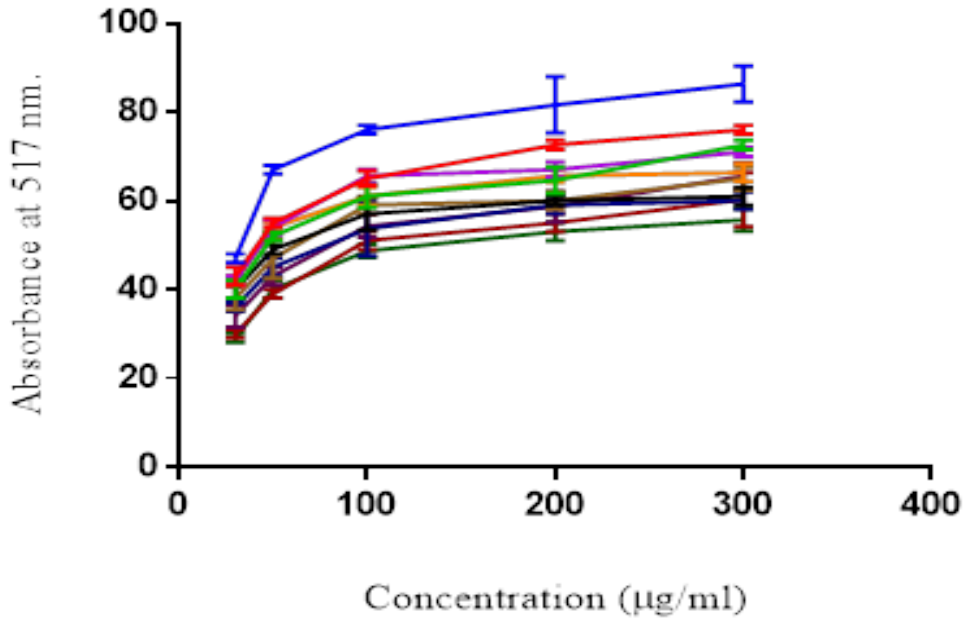

Figure 4.9: DPPH free radical scavenging of 10 varieties extracts of *Abelmoschus esculentus* and Standard.

Symbols: (—) stands for AA, (—) for SB, (—) for MC, (—) for OAI, (—) for OAL, (—) for SS, (—) for DC, (—) for BI, (—) for SH, (—) for KB and (—) for IB

In this study, the DPPH free radical scavenging activity of 10 varieties okra premature pod methanolic extract showed the value ranged from 55.66 to 76.00 (% of inhibition) and IC_{50} value ranges were from 40.32±2.91 to 97.28±1.32 at the concentration of 30-300 µg/ml while the Acarbose as standard value ranges were 47.00 to 86.33 (% of inhibition) and IC_{50} value was 27±1.8 at the concentration of 30-300µg/ml. In the DPPH free radical scavenging activity assay Shamol Bangla okra pod extract showed the highest value 76 (% of inhibition) and the IC_{50} value were 40.32±2.91 at the concentration of 300 µg/ml among the 10 varieties extracts of *A. esculentus*. The different concentration of sample and standard exhibited antioxidant activities in a dose dependent manner on the DPPH radical scavenging assay. Positive DPPH test indicated that the samples were free radical scavengers. The scavenging activity of 10 varieties extracts of *A. esculentus* was moderate when compared to standard vitamin C. This value range is mostly similar than the other reported values. Alam *et al*. (2017) reported that the plant methanolic extract were showed mild antioxidant activity (IC_{50} of the extraction is 499.17 µg/ml whereas IC_{50} of Ascorbic Acid is 16.407 µg/ml) in DPPH free radical scavenging activity. They also noted that *A. esculentus* has various beneficial properties, for that reason more researches should be performed to find out the impacts of this plant more correctly. Ayushi *et al*. (2016) reported that the IC_{50} values of the Extract and the Standard were found to be 53.96 and 32.25 µg/ml respectively, which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent. They also noted that the aqueous extract of *A. esculentus* leaves, having therapeutically important phytochemicals, could be developed not only as an antiaging agent but also as an agent for controling oxidative stress due to diabetic diseases. Kishore *et al*. (2011) investigated that the DPPH of taulang honey was expressed greater antioxidant (53.06±0.41 mg ascorbic acid equivalents per g) for the presence of higher phenolic content. Maltas *et al*. (2011) and Maltas & Yildiz (2012) studied that the methanolic extract of *ginko biloba* demonstrated higher antioxidant activity in DPPH test due to presence of its higher phenolic and flavonoids content 76.0±5.2 mgGAE/g dry weight and 1160.79 µg/g respectively. Graham *et al*. (2017) reported that the DPPH percentage of inhibition range were 46.38 to 64.00 (%) among the five okra genotypes. Islam *et al*. (2016) investigated that the methanolic extract of fruit of *Ficus recemosa* demonstrated the moderate antioxidant activity IC_{50} value were 42.80±1.78 µg/ml in the DPPH assay. Mamun *et al*. (2016) reported that the DPPH

assay showed the strong antioxidant activities where IC_{50} value was 5.1±0.9 µg/ml in the acetone extract of local variety of *Allium sativum.*

4.3.2.2.4 ABTS Free Radical Scavenging Activity

In the present analysis, the scavenging capacities of 10 varieties extracts of *Abelmoschus esculentus* for the ABTS radical were measured and compared with vitamin C. As shown in Table 4.4 the scavenging effect of extracts increased with increasing concentration. The extracts exhibited moderate scavenging properties when compared with vitamin C. At 400 µg/ml concentration methanol extract showed the highest percent of free scavenging capacity not equal to that of the standard ascorbic acid activity. Percent scavenging capacity of different extracts at 400 µg/ml was lower compared with that of AA. The IC_{50} values of different extracts were in the order of SB>MC>OAI>OAL >SS>DC>B1>SH>KB>IB (Table 4.6).

Table 4.4: ABTS free radical scavenging ability of standard and 10 varieties extracts of *Abelmoschus esculentus* and AA (Standard).

Conc.	% of inhibition										
µg/ml	AA	SB	MC	OAI	OAL	SS	DC	B1	SH	KB	IB
30	35.35±1.6	31.32±1.3	23.32±1.3	20.15±.0.21	18.52±0.4	16.35±1.31	14.35±0.58	13.09±0.46	12.16±0.83	11.73±0.59	10.03±1.35
50	51.47±2.2	47.61±1.5	44.61±1.5	35.28±1.1	24.47±0.6	18.39±1.17	16.16±1.36	15.42±1.25	14.59±1.74	13.62±0.74	12.19±0.79
100	80.81±1.1	59.52±1.7	55.52±1.7	49.36±0.9	40.55±1.1	31.01±1.8	29.48±1.42	28.73±1.14	27.61±0.86	26.17±1.42	15.38±1.48
200	85.27±1.5	70.15±1.8	61.15±1.8	58.41±1.3	56.13±1.3	55.52±1.6	53.39±0.82	52.79±1.61	45.92±1.58	40.83±1.26	29.25±1.63
400	93.14±1.2	**79.26±1.3**	67.26±1.3	63.24±1.6	60.63±1.7	57.16±1.2	55.71±1.26	53.47±1.92	51.48±1.79	47.27±1.84	45.39±1.92
IC_{50}	**22.35±1.8**	**53.12±2.1**	64.37±3.8	67.38±4.9	89.36±3.2	91.17±1.9	123.55±2.47	142.31±2.79	159.62±3.82	186.47±3.29	208.52±4.77

NB: Results are expressed as mean ± SEM (n=3)

Table 4.5: NO free radical inhibition activity of 10 varieties extracts of *Abelmoschus esculentus* and CA (Standard).

Conc.	% of inhibition										
(µg/ml)	CA	SB	MC	DC	OAI	OAL	SS	B1	KB	SH	IB
25	35.94±1.2	17.57±0.9	17.46±0.3	14.16±0.7	15.58±0.9	13.49±1.0	11.88±1.3	12.64±0.7	10.74±1.4	17.33±1.8	9.92±1.2
50	47.52±1.6	23.38±2.1	24.32±0.4	14.52±0.1	16.23±1.2	13.72±0.4	12.57±0.9	13.39±1.4	11.28±0.9	18.75±1.3	10.85±0.6
100	52.84±1.9	38.59±2.7	43.29±1.2	23.85±0.7	25.52±1.9	21.64±1.6	17.52±0.5	20.13±1.2	15.69±0.5	27.38±0.4	13.66±0.4
200	69.58±0.6	54.26±2.4	53.83±1.6	46.69±0.3	48.29±1.2	44.79±1.8	38.68±1.3	43.38±2.1	35.73±1.3	52.46±1.9	33.11±1.7
400	81.36±1.1	60.93±1.6	63.37±1.9	52.42±1.1	54.88±1.5	50.68±1.1	43.43±1.6	48.26±0.8	39.46±1.9	58.35±1.4	35.49±1.2
800	89.49±0.9	62.52±1.7	68.59±1.5	54.27±1.6	56.55±1.9	53.42±0.9	45.51±2.0	50.42±2.3	43.37±2.0	60.63±1.6	39.91±1.8
IC_{50}	**28.31±2.96**	67.73±2.8	**47.26±2.8**	179.97±2.1	167.85±2.7	195.37±2.3	202.28±3.6	198.19±2.9	225.71±4.6	83.94±3.1	391.52.±3.7

NB: Results are expressed as mean ± SEM (n=3)

The ABTS free radical scavenging activity of 10 varieties okra premature pod methanolic extract showed the value ranged from 45.39 to 79.26 (% of inhibition) and IC_{50} value ranged were 53.12±2.1 to 208.52±4.77 at the concentration of 30-400 µg/ml while the Acarbose as standard value ranged were 35.35 to 93.14 (% of inhibition) and IC_{50} value were 22.35±1.8 at the concentration of 30-400µg/ml. In the ABTS free radical scavenging activity assay Shamol Bangla okra pod extract showed the highest value 79.26 (% of inhibition) and the IC_{50} value were 53.12±2.1 at the concentration of 400 µg/ml among the 10 varieties extracts of *A. esculentus*. Positive ABTS test indicated that the samples were free radical scavengers. The report of ABTS on okra is not available. Islam *et al*. (2016) investigated that the ethanolic extract of fruit of *Ficus recemosa* demonstrated the high-level antioxidant activity IC_{50} value was 65.15±3.4 µg/ml in the ABTS assay. Mamun *et al*. (2016) reported that the ABTS assay showed the strong antioxidant activities where IC50 value were 11.3±0.2 (µg/ml) in the acetone extract of local variety of *Allium sativum.* Long *et al*. (2000) reported that the scavenging of the ABTS derived nitrogen-centred radical cation ($ABTS^{\bullet+}$) was employed to analogize the total antioxidant activities of different seasonings utilized in Asian cooking. The ABTS assay IC_{50} value was 14.93±0.91 (µg/ml). The outcomes were demonstrated as Trolox comparable antioxidant capacity (TEAC).

4.3.2.2.5 Nitric Oxide (NO) Scavenging Activity

In the present investigation, the different concentration of 10 varieties extracts of *Abelmoschus esculentus* exhibited antioxidant activities in a dose dependent manner (shown in Table 4.5) on the NO radical scavenging assay. 10 varieties methanol extracts of *Abelmoschus esculentus* caused a medium dose-dependent inhibition of nitric oxide with an IC_{50} of 47.26±2.8 µg/ml. The scavenging activity of Catechin showed IC_{50} values of 28.31±2.96 µg/ml (Table 4.5).

The different concentration of 10 varieties extracts of *A. esculentus* exhibited antioxidant activities in a dose dependent manner (shown in Table 4.5) on the NO radical scavenging assay. Positive NO test indicated that the samples were free radical scavengers. NO scavenging activity IC_{50} value ranged from 47.26±2.8 to 391.52±3.7 µg/ml. 10 varieties methanol extracts of *A. esculentus* caused a medium dose-dependent inhibition of nitric oxide with an IC_{50} of 47.26±2.8 µg/ml. The scavenging

activity of Catechin showed IC_{50} values of 28.31±2.96 µg/ml (Table 4.5). In the present work, the IC_{50} value and % of Inhibition (from 39.91±1.8 to 68.59±1.5) are within the range of other reported values. Ayushi *et al*. (2016) reported that NO radical Scavenging Activity exhibits the results of NO radical scavenging activity of both Extract and the Standard (at a concentration of 80 µg/ml) where the inhibitions percentage were 77.59% (IC_{50}: 59.15 µg/ml) in Extract and 85.99% (IC_{50}: 50.19 µg/ml) in Standard. As an antioxidant, they have confirmed that the leaves extract of *A. esculentus* is very much effective. Islam *et al*. (2016) studied that the ethanolic extract of fruit of *Ficus recemosa* showed the highest antioxidant activity IC_{50} value which was 140.3±2.35 µg/ml in the NO (nitric oxide) assay.

Table 4.6: Quantitative analysis of Phytochemicals and antioxidants values of 10 varieties of okra.

Variety	Phytochemicals				Antioxidants				
	Total phenol (mg of GAE/g extract dw)	Total flavonoid (mg of CAT/g extract dw)	Total flavonol (mg of QU/g extract dw)	Total proanthoc-yanidins (mg of CAT/g extract dw)	IC $_{50}$ (µg/ml)			Values of 160µg/ml	Values of 120µg/ml
					DPPH	ABTS	NO	TAC	FRAP
SB	11.37±0.39	9.88±0.09	5.75±0.09	6.86±0.52	40.32±2.91	53.12±2.1	67.73±2.8	1.285±0.013	1.293±0.019
MC	10.60±0.24	9.56±0.57	4.25±0.1	5.92±0.47	42.32±3.0	64.37±3.8	47.26±2.8	0.942±0.025	1.123±0.022
OAL	9.80±0.13	8.95±0.87	4.53±0.05	5.60±0.42	54.16±2.9	89.36±3.2	195.37±2.3	0.975±0.145	0.977±0.015
SH	8.84±0.34	7.60±0.68	3.86±0.17	5.11±0.58	85.36±2.2	159.62±3.82	83.94±3.1	0.883±0.012	0.884±0.127
OAI	10.21±0.17	9.38±0.82	4.61±0.14	7.25±0.27	52.65±1.81	67.38±4.9	167.85±2.7	1.023±0.171	1.101±0.163
SS	9.62±0.04	8.50±0.55	4.15±0.08	5.84±0.24	57.61±1.9	91.17±1.9	202.28±3.6	0.903±0.053	0.908±0.085
IB	8.64±0.64	6.70±0.88	3.91±0.12	5.48±0.36	97.28±1.32	208.52±4.7	391.52.±3.7	0.987±0.028	1.179±0.016
B-1	8.92±0.61	8.02±0.92	4.44±0.08	6.09±0.14	77.37±1.35	142.31±2.79	198.19±2.9	0.809±0.055	0.801±0.169
KB	8.72±0.05	7.03±0.12	2.57±0.11	4.57±0.11	95.23±1.21	186.47±3.29	225.71±4.6	0.894±0.072	0.897±0.155
DC	9.10±0.04	7.81±0.26	3.26±0.09	6.65±0.59	70.16±2.18	123.55±2.47	179.97±2.1	0.892±0.058	0.891±0.038

NB: Result are expressed as mean±SEM (n=3), GAE= Gallic Acid Equivalent, CAT= Catechin and QU=Quercetin.

From the results of phytochemicals and antioxidants study in above, it can be established that;

I. Okra obtains significantly high levels of total phenols, flavonoids, moderate content of flavonol, proanthocyanidin and a good source of natural antioxidants, which will habituate to the health of consumers.

II. There was high variability with consideration to TPCs, TFCs, flavonol, proanthocyanidin and antioxidants activity in the various accessions utilized in the study.

III. As yield higher, breeders can also investigate the possibility of breeding for high phytochemical and antioxidants containing varieties. The strong free radical scavenging activity of *Abelmoschus spp* requires further analyses for the isolation and identification of other bioactive compounds inherent in this species.

IV. The presence of different phytochemicals and antioxidants in the tested plant exibits that Shamol Bangla is the nutrient rich okra among the 10 varieties that may be a good choice for extraction of health supplements for various complications.

4.3.2.3 Quantitative analysis of medicinal properties of okra

4.3.2.3.1 Brine Shrimp Lethality Bioassay

In this bioassay, the mortality rate of brine shrimp was found to increase with the increase in concentration of the test sample. So, it was observed that the extracts exhibited brine shrimp low cytotoxicity (mild/slightly toxic) in a dose dependent manner (Table 4.7). The value of LC_{50}, indicates the low cytotoxic effect of okra pod extracts. Extracts lowest LC_{50} value of brine shrimp lethality was found in Mahira Cross 788.22 µg/ml among the 10 varieties of okra and Standard cytotoxic agent gallic acid was found to exhibit higher cytotoxicity with a lower LC_{50} value 16.69 µg/ml, maximum mortalities GA (100%) were observed at a concentration of 400 µg/ml (Table 4.7). Above range value was showed consistent as potent for some varieties and some varieties inconsistency was also found among the 10 varieties extract of okra compared to the other reported values. Gupta *et al*. (1996) reported that the LC_{50} of 1000 µg/ml was estimated as potent (active). Another report was noticed by Meyer *et al*. (1982) that the LC_{50} value of less than 1000 µg/ml is toxic while LC_{50} value of greater than 1000 µg/ml is non-toxic. Olowa & Nuñeza *et al*.

(2013) reported that the extracts of *L. camara*, *C. odorata*, and *E. hirta* were potent against the brine shrimp with LC_{50} values of 55, 10, and 100 ppm (µg/ml), respectively. Biswas *et al*. (2014) reported that the LD_{50} value of methanol extracts and petroleum ether fraction of Pumpkin (*Curcurbita maxima*) seed were determined and found to be 31.70 ppm and 21.95 ppm respectively in brine shrimp lethality bioassay. Rajeh *et al*. (2010) reported that the *E. hirta* extracts had LC_{50} values of 0.71, 0.66, 0.41 and 0.03 mg/ml for stems, leaves, roots and flowers, respectively against *Artemia salina*. A general bioassay that appears capable of detecting medium range of bioactivity present in pod crude extracts is the brine shrimp lethality bioassay (BSLT). The technique is easily mastered, of little cost, and uses small amount of test material. However, in this study the extracts of most of the varieties (SB, MC, OAL, SH, OAI and KB) were showed slightly toxic and some variety (SS, IB, B1 and DC) showed non-toxic range values among the 10 varieties of okra. It is noted that the rich (higher value) in presence of phytochemicals and antioxidant properties the Shamol Bangla variety was also showed the slightly toxic LC50 value 934.57 µg/ml may be a good source for production of new drugs for various complications among the 10 varieties premature pod extract of *A. esculentus*. For some varieties values inconsistency, further study is needed for more accuracy in future. Finally, it can be concluded that the methanolic extracts of 10 varieties of extract tested showed a little bit (mild toxic/slightly toxic) toxicity against brine shrimp larvicidal activity (Table 4.7). Therefore, the uses of this plant species in traditional medicine and pod can be used as edible fruit without hazard. The result acquired from the brine shrimp lethality bioassay of premature pod methanolic extracts of *A. esculentus* can be utilized as a preliminary guide for the isolation of cytotoxic compounds.

Table 4.7: LC_{50} value for 10 varieties extracts of *A. esculentus*

Conc. µg/ml	Log conc.	% of mortality											LC_{50} (µg/ml)								
		SB	MC	OAL	SH	OAI	SS	IB	B1	KB	DC	GA	SB	MC	OAL	SH	OAI	SS	IB	B1	KB	DC	GA
10	0.99	0	0	0	0	0	0	0	0	0	0	46.66	934.57	**788.22**	876.75	889.90	928.43	1135.23	1035.83	1034.53	890.74	1498.74	**16.69**
100	1.99	16.66	23.33	23.33	20	23.33	20	13.33	20	16.66	20	66.66											
200	2.30	33.33	33.33	30	36.66	33.33	30	30	33.33	36.66	26.66	83.33											
400	2.60	36.66	40	36.66	36.66	33.33	30	36.66	33.33	36.66	33.33	100											
800	2.90	40	53.33	43.33	40	43.33	40	36.66	40	40	33.33	100											

NB: GA=Gallic Acid

4.3.2.3.2 Antibacterial activity

The antibacterial activity of the crude methanol extract was tested against eight bacteria at concentrations of 300, 600 and 900 µg. Standard antibiotic disc Azithromycin (15 µg/disc) was used for comparison. The results obtained are shown in Table 4.8 and Figure 4.10 (a – h).

Extract of each variety showed antibacterial activity against 8 strains of bacteria and highest zone was 18 mm (at doses of 900 µg/ disc) showed in Shamol Bangla (SB). It is the dose dependent manner, when the concentration was increased the zone of inhibition was increased of every dose of all variety. It is consistent with the brine shrimp lethality bioassay, there was the lowest IC_{50} value 788.22 (µg/ml) showed the cytotoxicity and this cytotoxicity detect the accountability for the presence of phytochemicals and antioxidants such as phenols, flavonoids, alkaloids etc. in the pod extract of okra.

From the above experiment it has been found that the extracts might have some compounds, which are responsible for the microbial activity. Premature okra pods contain phenolics, flavonoids, vitamin C and the qualitative experiment of methanol extract showed the presence of saponins, flavonoids, terpenoids, tannins and Glycosides. These compounds have been reported to possess antibacterial activity (Aziz *et al*., 1998; El-Gammal *et al*., 1986; Cowan, 1999). Minerals demonstrated significant antibacterial activity (Williams & Haydel, 2010) against both Gram positive and Gram negative bacteria is an indication of broad spectrum of activity and thus can be used to source antibiotic substances for drug development that can be used in the control of these bacterial infections. Hasan *et al*. (2017) reported that the strong antibacterial activity was displayed by methanol, ethanol and petroleum ether extract of fruit of *Ficus racemosa* against most of the tested bacteria with the zone of inhibition of 13 to 17 mm at a concentration of 600 µg/disc. Wilkins *et al*. (1972) reported those strains of *P. anaerobius* with zone diameters larger than 16 mm could be considered susceptible to penicillin. Kawsar *et al*. (2015) reported that the growth of inhibition zone observed against Gram-positive *S. aureus* by two test chemicals (5'-O-N-acetylsulfanilyl-2',3'-di-O-lau- royluridine and 5'-O-N-acetylsulfanilyl-2',3'-di-O-pivaloyluridine) and highest zone was 14 mm at 200 µg dw/disc. Jasthi (2019) was mentioned that the green peel ethanolic extract demonstrated maximum

inhibitory effect on all tested bacteria compared to the acetone and ethanolic extracts with seeds and white peel. Oloketuyi (2017) was observed that the aqueous and ethanolic extracts of the four samples of *A. esculentus* seeds showed broad range of inhibitory activity (range was 14-30 mm) against the test bacteria especially *L. monocytogenes* and recommended that the *A. esculentus* seed has a great potential as effective antilisterial and antibacterial activity. Bello *et al*. (2015) observed that the ethanolic seed extract of *A. esculentus* showed maximum inhibitory effect on all the tested bacterium except Pseudomonas species compared to the aqueous extract.

Mostafa *et al*. (2014) investigated that all the six plant (*Tamarindus indica, Azadirachta indica, Cucumis sativus, Eucalyptus camaldulensis, Switenia mahagoni, and Psidium guajava*) species (not all extracts) demonstrated a medium antibacterial activity against a wide variety of gram positive and gram negative bacteria (no. of bacteria-11) at a concentration of 500 µg/disc where the zone of inhibition range were 9-19 mm. in the hexane, methanol, and water extracts. In this experiment, Kanamycin (30 µg /disc) was utilized as standard antibacterial agent. Antibacterial activity of *Euphorbia hirta* L. leaf, flower, stem and root extracts showed inhibition larger zones against Gram positive bacteria than Gram negative bacteria. Inhibition zones ranged between 16–29 mm (Rajeh *et al*., 2010). Antibacterial activity (moderate level) of premature pods of methanolic extracts of *A. esculentus* 10 varieties have detected the significant activity showed against 8 strains of bacteria and highest zone was 18 mm in support to the fact that extracts phytochemicals having antimicrobial activity. From the above discussion we can say that Shamol Bangla (SB) variety of *A. esculentus* premature pods extract might be used as best source of antibacterial agent among the 10 tested varieties of okra.

Table 4.8: *In vitro* Antibacterial activity of methanolic extract of 10 varieties premature pods of okra (*A. esculentus*) and Azithromycin.

Bacterial strain name	Zone of inhibition (mm) Dose (µg/ disc)																													
	SB			SH			MC			OAI			OAL			DC			SS			B1			KB			IB			Azithro-mycin
	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	900	600	300	15
Strepto coccus	16	14	13	15	14	13	13	12	11	15	13	11	14	12	11	14	12	10	13	12	10	14	13	11	12	11	9	13	12	10	18
Bacillus subtilis	14	13	11	15	13	11	13	12	10	13	12	10	14	12	11	13	11	9	13	11	9	13	12	11	14	13	11	14	11	10	15
Staphylococcus aureus	15	14	12	14	13	12	17	14	14	15	12	11	14	11	10	13	12	10	14	12	11	13	11	10	14	13	10	13	12	11	17
Shigella boydii	15	13	12	14	13	11	15	14	12	17	15	14	15	12	11	14	13	11	13	12	11	14	13	11	14	12	11	14	13	11	17
Shigella flexneni	13	12	11	13	12	10	14	13	12	14	12	11	15	14	13	13	11	10	13	10	9	14	13	12	13	11	10	14	12	9	15
Salmonella typhi	12	11	10	14	13	12	13	12	9	13	12	10	14	12	10	15	14	13	11	10	9	14	13	12	13	12	9	14	12	10	15
Escherichia coli	**18**	17	15	15	13	12	14	12	10	15	14	11	15	13	12	14	12	10	15	14	12	15	13	12	15	14	11	15	12	11	**24**
Shigella dysenteriae	14	12	10	14	13	11	15	13	12	15	13	10	14	13	12	15	12	11	16	15	13	14	13	10	15	12	11	15	12	11	16

The antibacterial activity is shown below in Figure 4.10 (a - h):

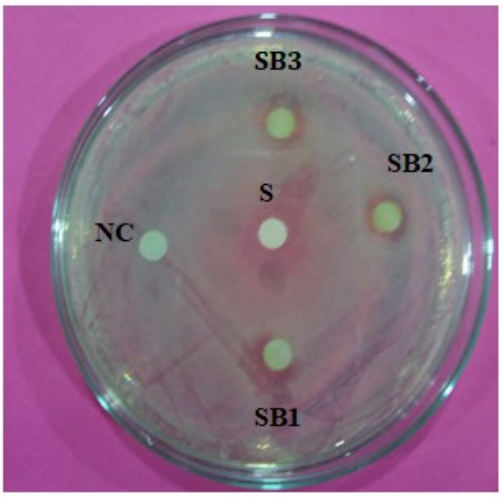

Figure (a): Antibacterial activity of SB against *Strepto coccus* (A^+)

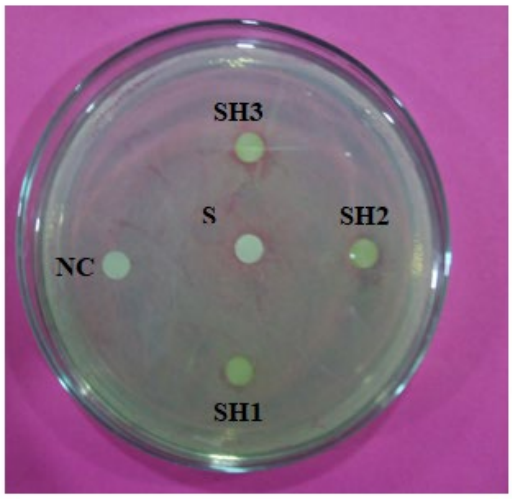

Figure (b): Antibacterial activity of SH against *Bacillus subtilis* (B^+)

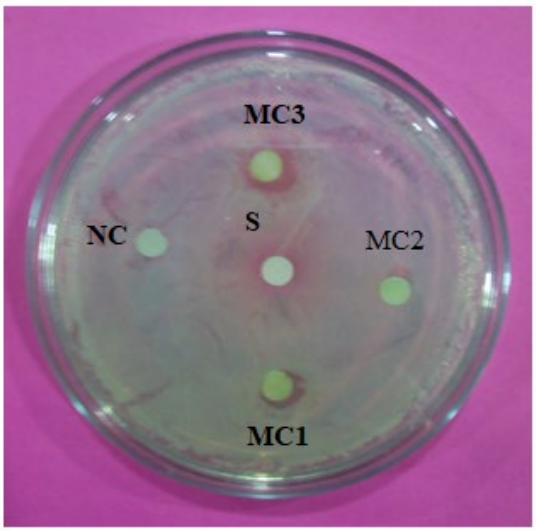

Figure (c): Antibacterial activity of MC against *Staphylococcus aureus* (D^+)

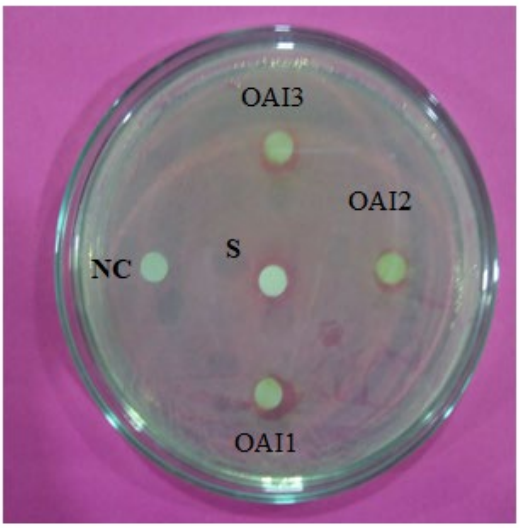

Figure (d): Antibacterial activity of OAI against *Shigella boydii* (E^+)

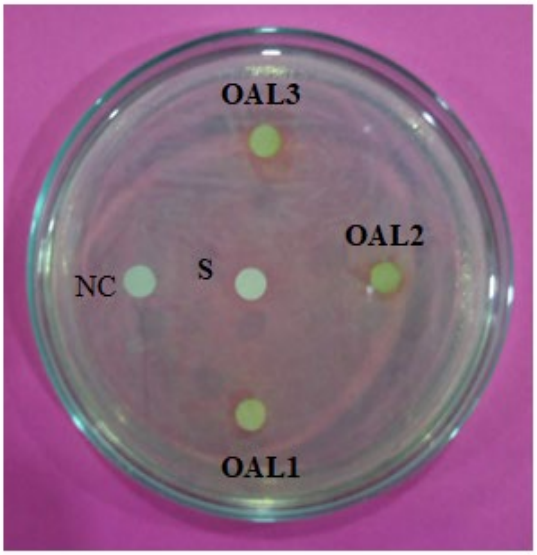

Figure (e): Antibacterial activity of OAL against *Shigella flexneni* (F^-)

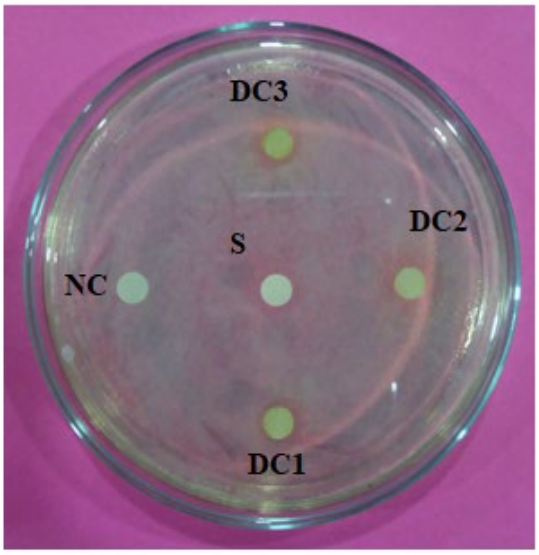

Figure (f): Antibacterial activity of DC against *Salmonella typhi* (G^-)

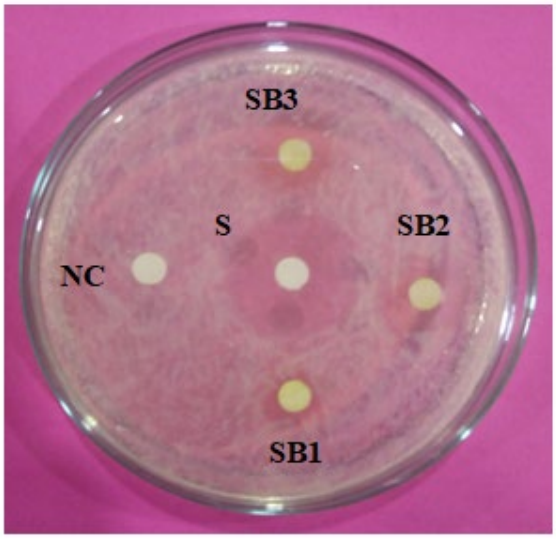

Figure (g): Antibacterial activity of SB against *Escherichia coli* (H^-)

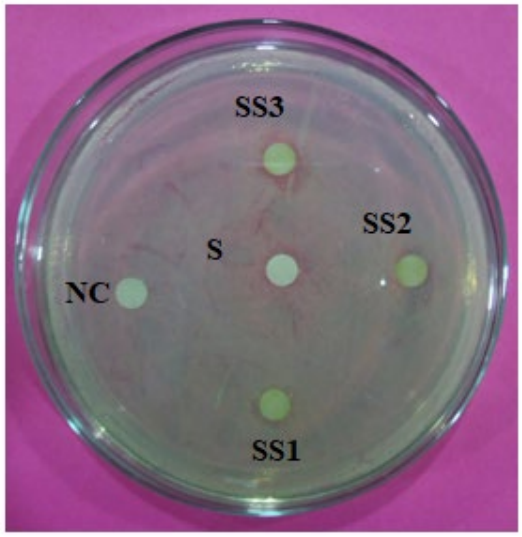

Figure (h): Antibacterial activity of SS against *Shigella dysenteriae* ($R5^-$)

[Note: S indicates standard (Azithromycin-15 µg/disc) and NC indicates negative (solvent) control. Number 1, 2, and 3 with the name of samples indicate 300, 600 and 900 µg of sample/disc, respectively.]

4.3.2.3.3 Antidiabetic activity

4.3.2.3.3.1 α-amylase inhibition assay: *In vitro* anti-diabetic activity DNSA (3, 5 dinitrosalicylic acid) method

Alpha-amylase inhibitory activities of 10 varieties okra (*A. esculentus*) premature pods extracts illustrated in Table 4.9.

According to Table 4.9 it was revealed that each extract indicated alpha-amylase inhibitory activity, which was in a dose-dependent mode. Moreover when explored with the concentration of 1000µg/ml, Shamol Bangla (SB) extract had the high-level inhibitory activity (69.75%) among the ten varieties extracts. However, compared with the commercial drug acarbose, no supremacy of extracts obtained higher inhibitory activities than acarbose in this study.

Shamol Bangla (SB) extract causes highest % of alpha-amylase inhibition at 1000µg/ml concentration as compare with another extract. At 1000µg/ml concentration 69.75±2.41, 48.91±2.35, 56.82±1.78, 54.65±2.62, 53.43±2.68, 51.62±2.19, 49.28±2.94, 47.62±1.96, 46.36±2.77, 42.59±2.92 percentage of α-Amylase inhibition were observed in 3,5-Dinitrosalicylic acid (DNSA) method by SB, DC, MC, OAI, SS, B1, OAL, SH, KB and IB respectively, where standard Acarbose exhibit 85.36±1.87 percentage inhibition.

Table 4.9: α-amylase inhibition activity of 10 varieties of Okra pod extracts in DNSA method.

CONC. (µg/ml)	**Percentage(%) of α-amylase inhibition**										
	3,5-dinitrosalicylic acid test										
	SB	DC	MC	OAI	SS	B1	OAL	SH	KB	IB	ACARBOSE
200	17.17±3.51	10.79±2.69	19.82±2.17	14.37±2.32	13.82±1.83	11.96±2.53	9.96±2.14	7.95±1.75	6.87±2.14	12.33±2.47	43.77±2.58
400	25.69±2.48	16.63±2.37	29.13±2.51	27.82±2.58	24.29±1.62	22.64±1.49	20.85±2.63	18.73±1.83	17.69±2.55	23.62±1.95	52.85±1.92
800	33.26±2.17	28.54±2.73	42.61±1.84	34.68±2.53	31.74±1.52	29.37±1.72	27.52±1.71	25.59±2.85	24.83±1.82	33.74±2.51	80.47±3.51
1000	**69.75±2.41**	48.91±2.35	56.82±1.78	54.65±2.62	53.43±2.68	51.62±2.19	49.28±2.94	47.62±1.96	46.36±2.77	42.59±2.92	**85.36±1.87**

4.3.2.3.3.2 α-glucosidase inhibition assay: *In vitro* anti-diabetic activity DNSA (3, 5 dinitrosalicylic acid) method

Alpha-glucosidase inhibitory activities of 10 varieties okra (*A. esculentus*) premature pods extracts are illustrated in Table 4.10. According to Table 4.10 it was revealed that each extract indicated alpha-glucosidase inhibitory activity, which was in a dose-dependent mode. Moreover when explored with the concentration of 1000µg/ml, Shamol Bangla (SB) extract had the high-level inhibitory activity (73.48%) among the ten varieties extracts. However, compared with the commercial drug acarbose, no supremacy of extracts obtained higher inhibitory activities than acarbose in this assay.

Shamol Bangla (SB) extract causes highest % of alpha-glucosidase inhibition at 1000µg/ml concentration as compare with another extract. At 1000µg/ml concentration 73.48 ± 1.75, 60.14±1.27, 58.51±2.38, 57.25±2.25, 55.46±2.27, 53.21±2.58, 52.56±2.26, 51.73±1.22, 50.11±2.43, 46.11±2.53 percentage of α-glucosidase inhibition were observed in 3,5-Dinitrosalicylic acid (DNSA) method by SB, MC, OAI, SS, B1, OAL, DC, SH, KB and IB respectively, where standard Acarbose exhibit 89.52±1.47 percentage inhibition.

Table 4.10: α-glucosidase inhibition activity of 10 varieties of okra extracts in DNSA method.

CONC. (µg/ml)	% of inhibition										
	3,5-dinitrosalicylic acid test										
	SB	DC	MC	OAI	SS	B1	OAL	SH	KB	IB	ACARBOSE
200	21.53±2.64	14.49±1.77	23.31±2.45	18.37±2.26	17.72±1.64	15.22±2.18	13.24±2.83	11.26±1.82	10.36±2.59	16.72±1.95	47.58±2.63
400	29.36±1.95	20.24±2.11	33.26±2.79	31.61±2.11	28.37±1.11	26.37±1.26	24.63±1.59	22.51±1.37	21.48±2.32	27.18±1.46	56.19±1.27
800	37.57±1.98	32.67±1.93	46.33±1.92	38.54±2.82	35.83±1.77	33.58±1.31	31.44±1.75	29.25±1.99	28.57±1.61	37.25±2.28	84.26±2.94
1000	73.48±1.75	52.56±2.62	60.14±1.27	58.51±2.38	57.25±2.35	55.46±2.27	53.21±2.58	51.73±1.22	50.11±2.43	46.11±2.53	89.52±1.47

In this study, the alpha amylase inhibition assay of 10 varieties okra premature pod methanolic extract showed the value ranged from 6.87±2.14 to 69.75±2.41 (% of inhibition) at the concentration of 200-1000 µg/ml while the Acarbose as standard value ranged were 43.77±2.58 to 85.356±1.87 (% of inhibition) at the same (200-1000 µg/ml) concentration. In the alpha amylase inhibition assay Shamol Bangla okra pod extract showed the highest value 69.75±2.41 (% of inhibition) at the concentration of 1000 µg/ml. In another way, the alpha glucosidase inhibition assay of 10 varieties okra premature pod methanolic extract showed the value ranged from 10.36±2.59 to 73.48±1.75 (% of inhibition) at the concentration of 200-1000 µg/ml while the Acarbose as standard value ranged were 47.58±2.63 to 89.52±1.47 (% of inhibition) at the same (200-1000 µg/ml) concentration. In the alpha glucosidase inhibition assay Shamol Bangla okra pod extract showed the highest value 73.48±1.75 (% of inhibition) at the concentration of 1000 µg/ml. From these two assays, alpha glucosidase inhibition of Shamol Bangla okra pod methanolic extract showed the highest value 73.48±1.75 (% of inhibition) at the concentration of 1000 µg/ml compared to the alpha amylase inhibition (DNSA method) among the 10 varieties okra pod extract and this inhibition is responsible for the phytochemicals whose presence was confirmed here in previous studies. The results of the both enzymes' inhibition activity were found in a dose-dependent manner. This inhibition (%) value ranges are mostly consistent compared to the other reported values.

Sabitha *et al*. (2012) reported that the alpha amylase inhibition by AAPP (peel) and AASP (seed) okra aqueous extract showed the value ranged from 34.89±0.1 to 87.57±0.3 and 30.5±0.3 to 80.06±0.2 (% of inhibition) where IC_{50} values were 132.63±0.16 and 147.23±0.21 respectively at the concentration of 50-250 µg/ml. In the alpha amylase inhibition assay AAPP (peel) okra aqueous extract showed the highest value 87.57±0.3 (% of inhibition) while the IC_{50} value were 132.63±0.16 at the concentration of 250 µg/ml. Another way, the alpha glucosidase inhibition by AAPP (peel) and AASP (seed) okra aqueous extract showed the value ranged from 30.8±0.6 to 88.7±0.2 and 29.3±0.2 to 80.9±0.4 (% of inhibition) where IC_{50} values were 142.69±0.32 and 150.47±0.28 respectively at the concentration of 50-250 µg/ml. In the alpha glucosidase inhibition assay AAPP (peel) okra aqueous extract showed the highest value 88.7±0.2 (% of inhibition) while the IC_{50} value was 142.69±0.32 at

the concentration of 250 µg/ml. From these two, alpha glucosidase inhibition assay AAPP (peel) okra aqueous extract showed the highest value 88.7±0.2 (% of inhibition) while the IC_{50} value was 142.69±0.32 at the same (250 µg/ml) concentration copmpared to the alpha amylase inhibition (DNSA method) of AAPP (peel) okra aqueous extract. The inhibitory effect in a concentration was dose dependent manner. Thus, the study confirmed the hypoglycemic effect in the AAPP and AASP aqueous extracts of *A. esculentus* and it was concluded that the results, gives a clear evidence that *A. esculentus* has antidiabetic activity.

Karim *et al*. (2014) reported that the alpha amylase inhibition by okra pod methanolic various extract showed the value ranged from 24.9±0.3 to 72.1±0.6 (% of inhibition) and IC_{50} value ranged were 104.54±1.35 to 148.56±1.90 at the concentration of 40-200 µg/ml while the Acarbose as standard value ranges were 39.3±0.1 to 88.7±0.2 ((% of inhibition) and IC_{50} value were 18.40±0.13 at the concentration of 10-50µg/ml. In the alpha amylase inhibition assay fresh okra pod extract showed the highest value 72.1±0.6 (% of inhibition) and the IC_{50} value were 104.54±1.35 at the concentration of 200 µg/ml. Another way, the alpha glucosidase inhibition by okra pod methanolic various extract showed the value ranges from 29.9±0.4 to 79.2±0.4 (% of inhibition) and IC_{50} value ranged were 70.48±0.91 to 133.01±1.33 at the concentration of 40-200 µg/ml while the Acarbose as standard value ranges were 45.6±0.3 to 93.3±0.1 (% of inhibition) and IC_{50} value were 13.70±0.20 at the concentration of 10-50µg/ml. In the alpha glucosidase inhibition assay blanched okra pod extract showed the highest value 79.2±0.4 (% of inhibition) and the IC_{50} value were 70.48±0.91 at the concentration of 200 µg/ml. From this two analyses, alpha glucosidase inhibition assay blanched okra pod methanolic extract showed the highest value 79.2±0.4 (% of inhibition) where the IC_{50} value was 70.48±0.91 compared to the alpha amylase inhibition (DNSA method) of fresh okra extract. It was recommended that Okra pods is going to be a potential anti-hyperglycemic agent even if eaten after cooking and suggested that regular intake of 100 g fresh okra as salad can vitally help to maintain blood sugar level of a moderate type 2 diabetic subject.

Bhutkar *et al*. (2018) reported the results demonstrated that the extract of *A. lebbeck* at a concentration of 1000µg/ml showed an inhibition (α-amylase) of 70.91%, whereas

the extracts of *B. aristata* and *M. pruriens* revealed an inhibition of 65.24% and 62.96 % respectively among the selected 6 plants aqueous extract at an equivalent concentration. The extracts of *C. roseus, M. fragrans* and *C. bonducella* showed a relatively less inhibitory reply for the α-amylase enzyme. So, they concluded that the results of the work clearly show the potential of the studied extracts to control hyperglycemia.

In the present study values are mostly consistent with the above results. So, this study confirmed the hypoglycemic effect in the premature pod methanolic extract of 10 varieties of okra and it can be concluded that the results gives a clear evidence that okra has antidiabetic activity. The Shamol Bangla variety showed the highest value of inhibition. Therefore, Shamol Bangla can be a good source for antihyperglycemic agent among the 10 varieties of okra to treat type 2 diabetes.

4.3.3 Quantitative analysis of Biochemical and Nutritional properties

4.3.3.1 pH of *A. esculentus* premature pods

pH of 10 varieties *of A. esculentus* premature pods are shown in the Table 4.11. It was observed the 10 varieties of *A. esculentus* premature pods P^H showed the pH scale value ranged from 6.5 to 6.8. The pH of *A. esculentus* premature pods was in the acidic range. The highest pH value was showed in Orka Anamika Local, BARI-1, Kolatia Bhendi (pH 6.8) and lowest in Shomy Hybrid, Dherosh Chamak (pH 6.5). Reports are not available. Therefore, the results indicate that the 10 varieties of *A. esculentus* premature pods P^H is mild/slightly acidic.

4.3.3.2 & 4.3.3.3 Moisture and ash content of *A. esculentus* premature pods

Moisture is necessary for most of the physiological reaction in plant tissue and if it is lack, life does not exist. The moisture contents were found to be varied in 10 varieties (SB, MC, OAL, SH, OAI, SS, IB, B-1, KB and DC) of *A. esculentus* premature pods 90.21, 90.43, 91.33, 90.55, 89.89, 89.47, 89.11, 90.41, 89.92 and 90.28 (g%) respectively as shown in Table 4.11. The highest moisture content was found in Shomy Hybrid 90.55%. The moisture content value ranged from 89.11 to 90.55 (g%) which is within the range value than the other reported values. Moisture content value ranged from 87.98 to 90.60 g/100g (Gemede *et al*., 2016). Also this is in accordance

with the finding of Gopalan *et al*. (2007) (89 g/100g) and (Nwachukwu *et al*., 2014) (88.47 g/100g). The moisture, contents were 82.53±1.60% (Roy *et al*., 2014) found in the leaves of *A. esculentus*.

Most of the inorganic constituents or minerals are present in ash. The highest ash content was measured in Shamol Bangla 10.647 (g%) premature pod of *A. esculentus* is given in the Table 4.11. The 10 varieties of *A. esculentus* premature pods Ash content showed the mean value ranged from 6.268 to 10.647 (g%) which is within the range value than the other reported values. The level of ash content was ranged from 5.37 to 11.30 g/100g (Gemede *et al*., 2016). The mean of ash content value ranged 7.19–9.63 g/100g (Adetuyi *et al*., 2011). Roy *et al*. (2014) reported that the ash content was found in the leaves of *A. esculentus* 18.48±0.03%. In this study, the results showed that the Shamol Bangla contains high ash content which indicates that the Shamol Bangla okra pods would provide essential valuable and useful minerals needed for body development.

Table 4.11: pH, moisture and ash content of *A. esculentus* premature pods

Name of Variety	P^H	Mean Value	
		Moisture (g %)	Ash (g %)
SB	6.6	90.21	**10.647**
MC	6.7	90.43	9.582
OAL	6.8	91.33	7.356
SH	6.5	**90.55**	6.268
OAI	6.6	89.89	8.489
SS	6.7	89.47	1.641
IB	6.7	89.11	8.397
B-1	**6.8**	90.41	8.456
KB	6.8	89.92	9.528
DC	6.5	90.28	10.636

4.3.3.4 Carbohydrate content of *A. esculentus* premature pods

Carbohydrate content of premature *A. esculentus* pod*s* are shown in Table 4.12 and Figure 4.11. Carbohydrates are specially present in the shape of mucilage (Kumar *et al*., 2009; Liu *et al*., 2005). Mucilage from okra also includes significant amounts of protein, carbohydrate, neutral sugars, minerals and other complicated polysaccharides

(Ndjouenkeu *et al*., 1996; Woolfe *et al*., 1977). In this study, the highest amount of carbohydrate was present in Shamol Bangla (SB) i.e 7.42% (g%) of *A. esculentus* premature pods. The 10 varieties premature pods carbohydrate content value ranged from 6.12 to 7.42 (g%) which is within the range value than other reported values. Approximately, the edible portion of the fruit carbohydrate contained 8.20 g/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978). The amalgamation of okra leaf per 100 g edible portion carbohydrate is 11.30 g/100g reported by (Benchasri, 2012a; VarmuDy, 2011; Gopalan *et al*., 2007). Gemede *et al*. (2016) investigated that the utilizable carbohydrate value varied from 36.66–50.97 (g/100g). The carbohydrate value was 7.45 g/100g (USFDA data base report). Sindhu and Puri, 2016 reported that the carbohydrate value was 7.6 g/100g. Nwachukwu *et al*. (2014) studied that the carbohydrate content was 7.05 g/100g. In another report of Nwachukwu *et al*., 2014; Idris *et al*., 2009, the leaf carbohydrate content were 27.54±0.27%. Roy *et al*. (2014) reported that the carbohydrate content were 7.03g/100g. The present value range was found to be most consistent with the other reported values which supported the present value and Shamol Bangla could be used as a good source for carbohydrate.

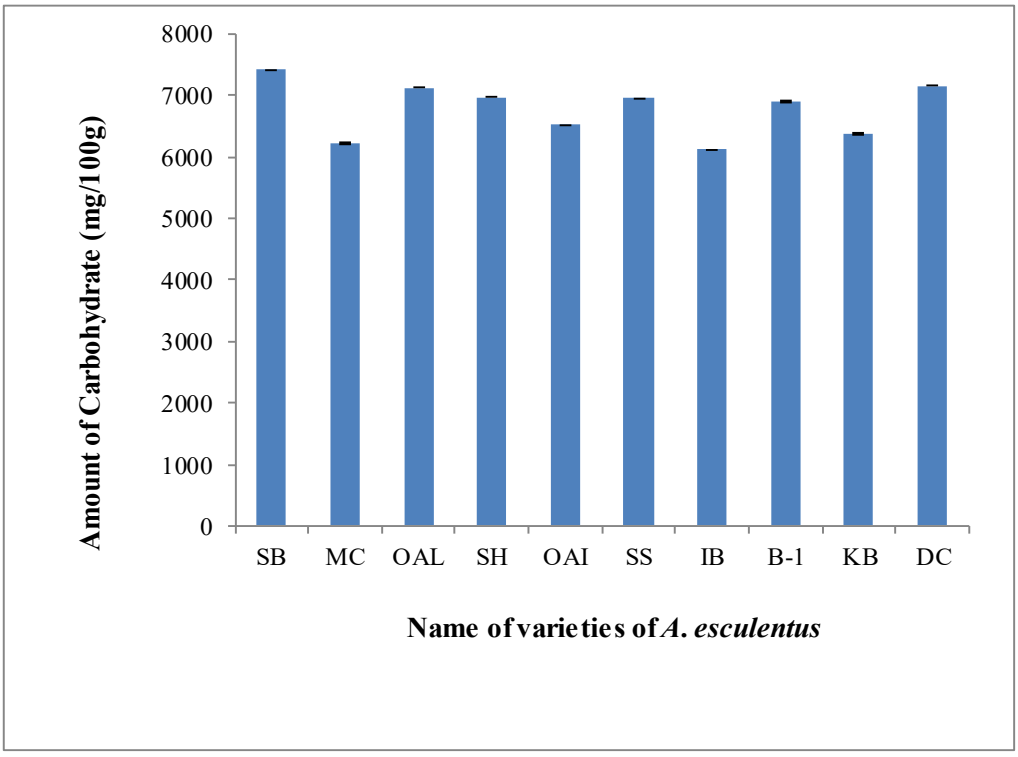

Figure 4.11: Carbohydrate contents of 10 varieties of *Abelmoschus esculentus*.

4.3.3.5 Water-soluble protein content of *okra* premature pods

The amounts of water-soluble protein present in the 10 varieties of *A. esculentus* premature pods are shown in the Table 4.12 and Figure 4.12. In this study, the highest amount of protein was present in Shamol Bangla (SB) i.e 2.15% (g%) of *A. esculentus* premature pods. The 10 varieties premature pods protein content value ranged from 0.95 to 2.15 (g%) which is within the range value than other reported values. Approximately, the edible portion of the fruit protein contained 2.10 g/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978). The composition of okra leaf per 100 g edible portion protein is 4.40 g/100g reported by (Benchasri, 2012a; VarmuDy, 2011; Gopalan *et al*., 2007). Gemede *et al*. (2016) investigated that the crude protein value ranged from 10.25–26.16 (g/100g). The protein content value was 2 g/100g (USFDA data base report). Sindhu & Puri, (2016) reported that the protein value was 2.0 g/100g. Nwachukwu *et al*. (2014) studied that the protein content was 2.56 g/100g. In another report of Nwachukwu *et al*., 2014; Idris *et al*., 2009, the leaf protein content was 7.63±0.06%. Roy *et al*. (2014) investigated that the protein content were 2.00 g/100g. The present value range found to be most consistent and supported with the other reported values and Shamol Bangla could be used as a good source for protein.

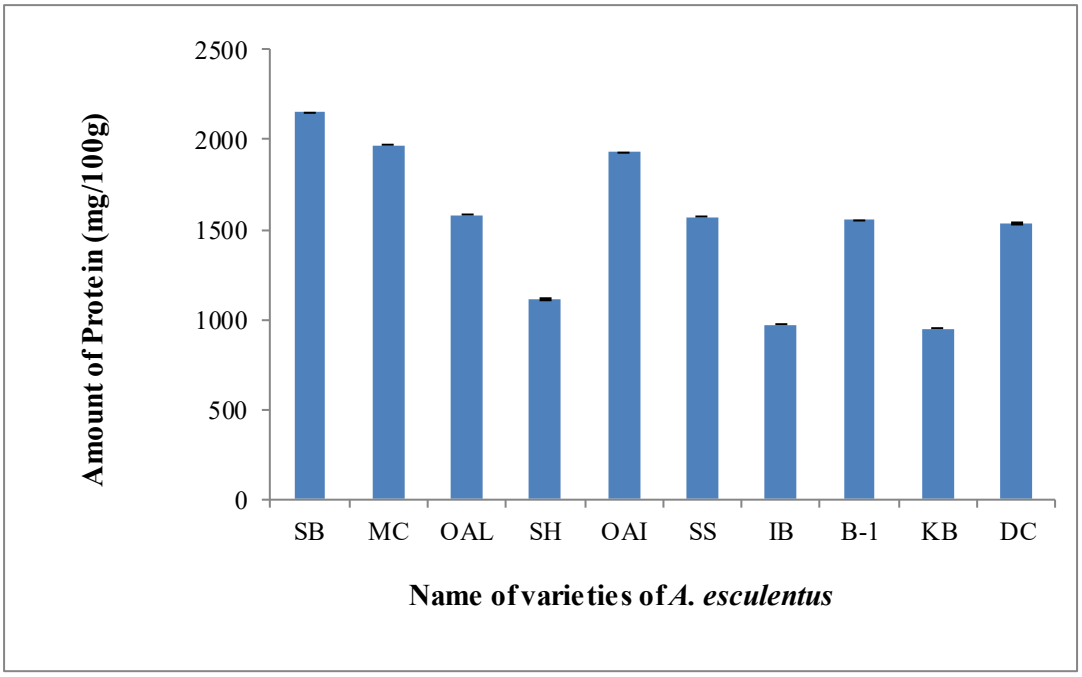

Figure 4.12: Protein contents of 10 varieties of *Abelmoschus esculentus*.

4.3.3.6 Lipid content of *A. esculentus* premature pods

Fats are concentrated form of energy and are important as carrier of certain fat-soluble vitamins such as, A, D, E and K. Lipid contents of 10 varieties *A. esculentus* premature pods are presented in Table 4.12 and Figure 4.13. In the present study, the highest amount of lipid was present in Sobuj Sathi (SS) i.e 0.11 (g%) among the 10 varieties premature pods of *A. esculentus*. The 10 varieties premature pods lipid content value ranged from 0.05 to 0.11 (g%) which is within the range value than other reported values. The okra protein contained 2.0% reported by (Dilruba *et al*., 2009; Arapitsas, 2008; Mays *et al*., 2007; Gopalan *et al*., 2007; Owolarafe & Shotonde, 2004; Lamont, 1999). Gemede *et al*. (2016) investigated that the lipid value ranged from 0.56–2.49 g/100g. The lipid content value was 0.19 g/100g (USFDA data base report). Sindhu & Puri, (2016) reported that the protein value was 0.1 g/100g. In the report of Nwachukwu *et al*., 2014; Idris *et al*., 2009, found that the leaf lipid content was 12.98±0.03%. Roy *et al*. (2014) studied that the lipid content were 0.10 g/100g. The present value range was found to be most consistent and supported with the other reported values. The present data clearly indicate that 10 varieties *A. esculentus* premature pods contained very little amount of lipid.

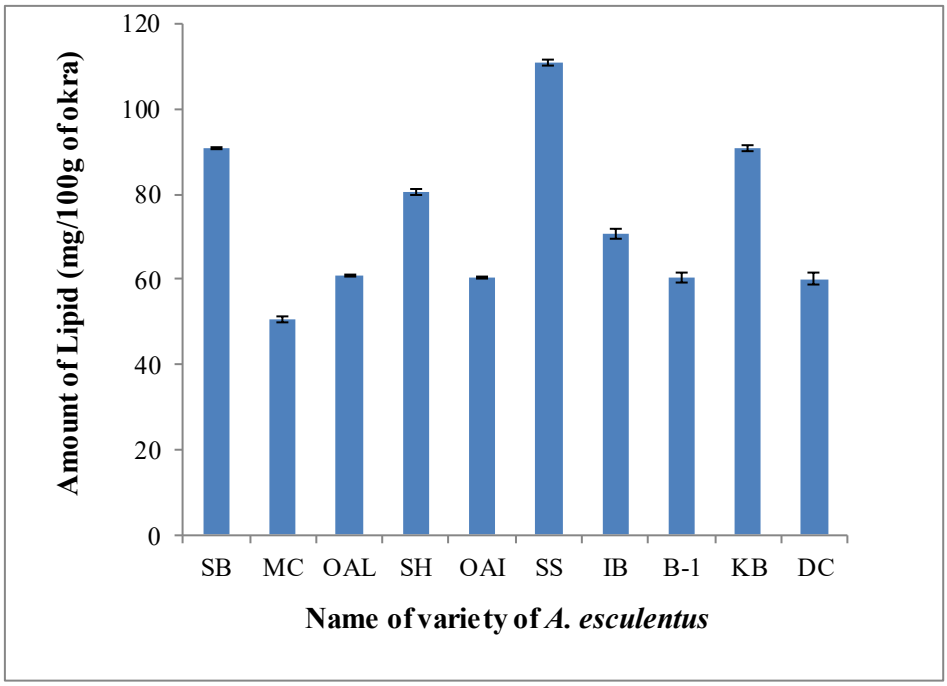

Figure 4.13: Lipid contents of 10 varieties of *Abelmoschus esculentus*.

4.3.3.7 Total soluble sugar content of *A. esculentus* premature pods

Total soluble sugar contents of 10 varieties *A. esculentus* premature pods are given in Table 4.12 and Figure 4.14. As shown in Table 4.12, the total soluble sugar content was found highest in the Dherosh Chamak (DC) i.e 1.28% (g%) among the 10 varieties premature pods of *A. esculentus*. The 10 varieties premature pods protein content value ranged from 0.73 to 1.28 (g%). Few reports are available to support the present study value. The total soluble sugar content value was 1.48 g/100g (USFDA data base report). Roy *et al*. (2014) investigated that the total soluble sugar content was 1.2 g/100g. The present value range was found to be most consistent and supported by the other reported values.

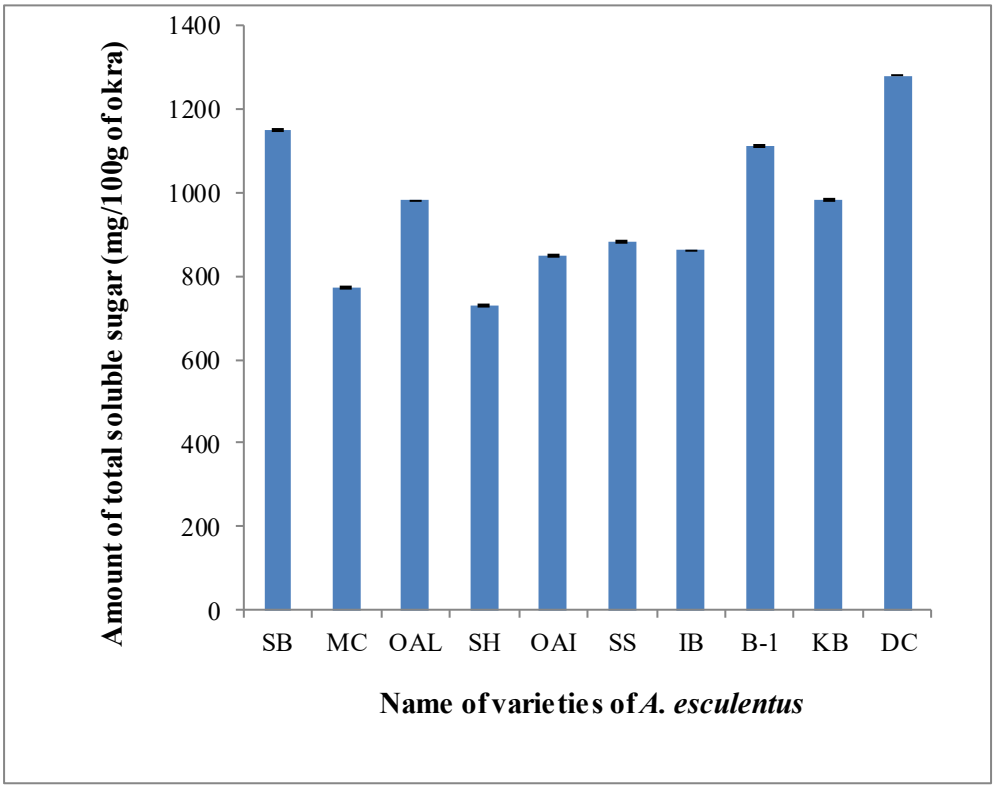

Figure 4.14: Total Soluble Sugar contents of 10 varieties of *Abelmoschus esculentus*.

4.3.3.8 & 4.3.3.9 Reducing and non-reducing sugar (sucrose) content of *A. esculentus* premature pods

The increases in reducing sugar were due to enzymatic conversion of strach to reducing sugar and also conversion of some non-reducing sugar (Gogoi *et al*., 2012). The reducing and non-reducing sugar (sucrose) contents of 10 varieties premature pods of *A. esculentus* are shown in Table 4.12, Figure 4.15 and 4.16.

The reducing sugar content was highest at the Dherosh Chamak i.e 0.81% (g%) among the 10 varieties premature pods of *A. esculentus* and the reducing sugar content of other 09 varieties were 0.70%, 0.48%, 0.74%, 0.34%, 0.62%, 0.60%, 0.61%, 0.78% and 0.71% (g per 100 g of premature okra pods) for SB, MC, OAL, SH, OAI, SS, IB, B-1 and KB respectively. The reducing sugar content value were varied from 0.34 to 0.81 (g%). Other reporters' value is not available.

The non-reducing sugar (sucrose) content present in the 10 varieties of *A. esculentus* premature pods are shown in the Table 4.12 and Figure 4.16. The non-reducing sugar (sucrose) content was highest at the Dherosh Chamak (DC) i.e 0.47% (g%) among the 10 varieties premature pods of *A. esculentus*. In the present study, the 10 varieties premature pods non-reducing sugar (sucrose) content value varied from 0.23 to 0.47 (g%) which is within the range value compared to other reported values. Roy *et al*. (2014) reported that the non-reducing sugar (sucrose) content was 0.40 g/100g. The present value range was found to be most consistent and supported with the other reported value and Dherosh Chamak could be used as a good source for non-reducing sugar (sucrose).

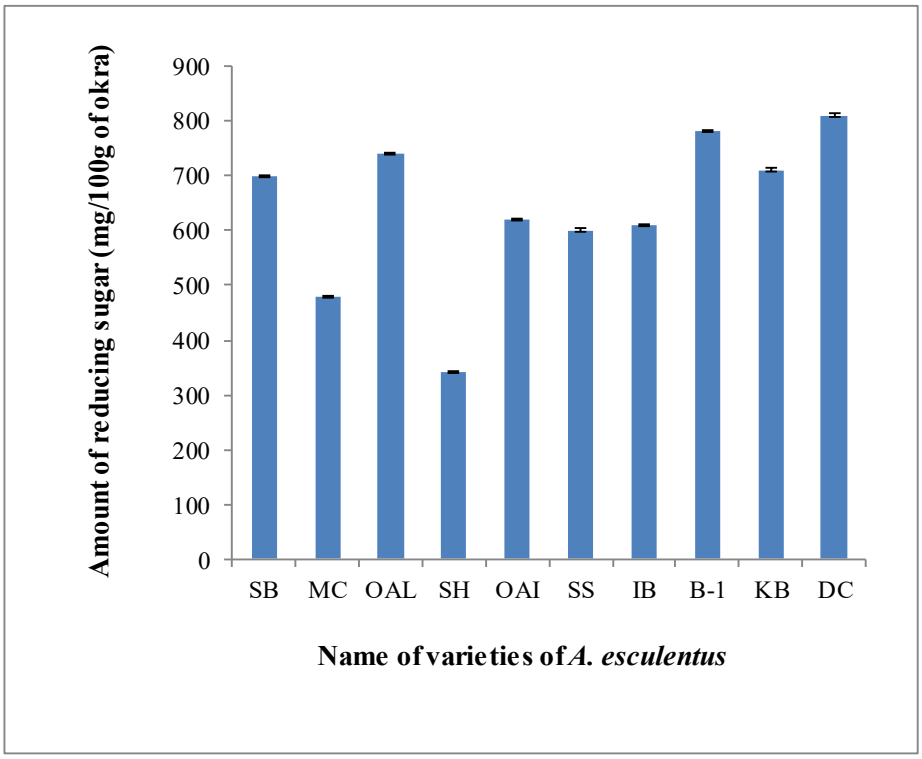

Figure 4.15: Reducing Sugar contents of 10 varieties of *A. esculentus*.

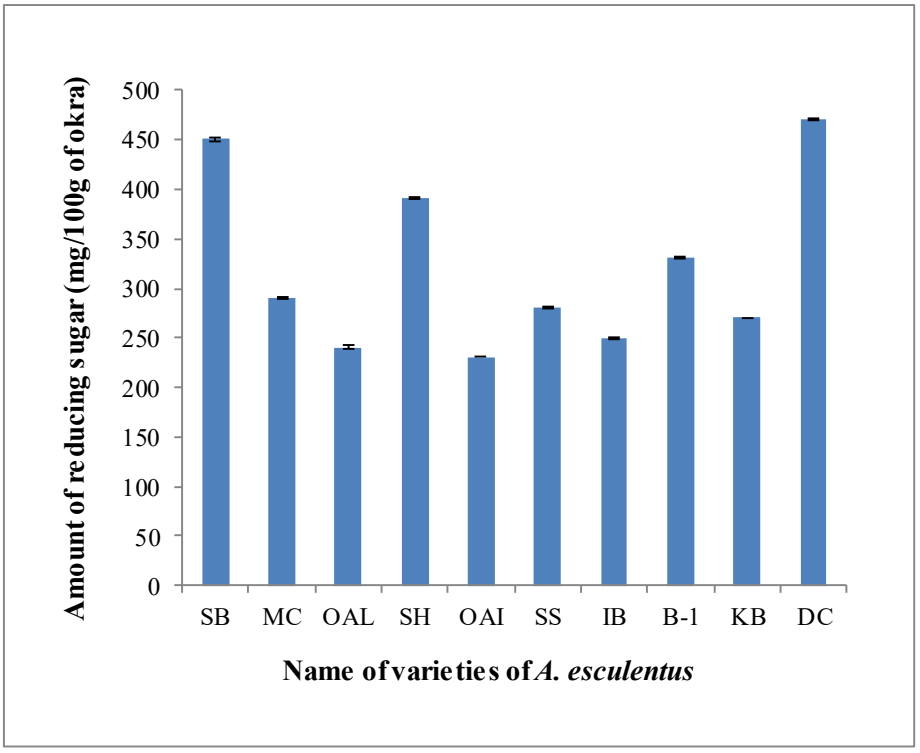

Figure 4.16: Sucrose (or Non-Reducing Sugar) contents of 10 varieties of *Abelmoschus esculentus*.

4.3.3.10 Vitamin –C content of *A. esculentus* premature pods

The amounts of vitamin-C present in 10 varieties of *A. esculentus* premature pods are given in the Table 4.12 and Figure 4.17. The Vitamin-C content was highest at the Shamol Bangla (SB) i.e 23.95 (mg/100g) among the 10 varieties premature pods of *A. esculentus*. The 10 varieties premature pods protein content value ranged from 15.42 to 23.95 (mg/100g) which is within the range value than other reported values. Approximately, the edible portion of the fruit vitamin-C contained 47.00 mg/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978). The constitution of okra leaf per 100 g edible portion vitamin-C is 59.00 mg/100g reported by (Benchasri, 2012a; VarmuDy, 2011; Gopalan *et al*., 2007). The value of the vitamin-C content was found 92 mg/100mg reported by (Dilruba *et al*., 2009; Arapitsas, 2008; Mays *et al*., 2007; Gopalan *et al*., 2007; Owolarafe & Shotonde, 2004; Lamont, 1999). Cook *et al*. (2000) studied that the vitamin-C content value ranged were 16 to 29 mg/100g and the value supported by Moyin-Jesu (2007), they suggested that the plant residues can be used for improving soil fertility, pod nutrients, root growth and pod weight of okra (*Abelmoschus esculentum* L). The vitamin-C content value was 23mg (28%)/100g (USFDA data base report). Sindhu & Puri (2016) reported that the vitamin-C value was 23 mg/100g. Roy *et al*. (2014) investigated that the vitamin-C content were 21.1 mg/100g. The present value range was found to be most consistent and supported by the other reported values and Shamol Bangla could be used as a good source for Vitamin-C.

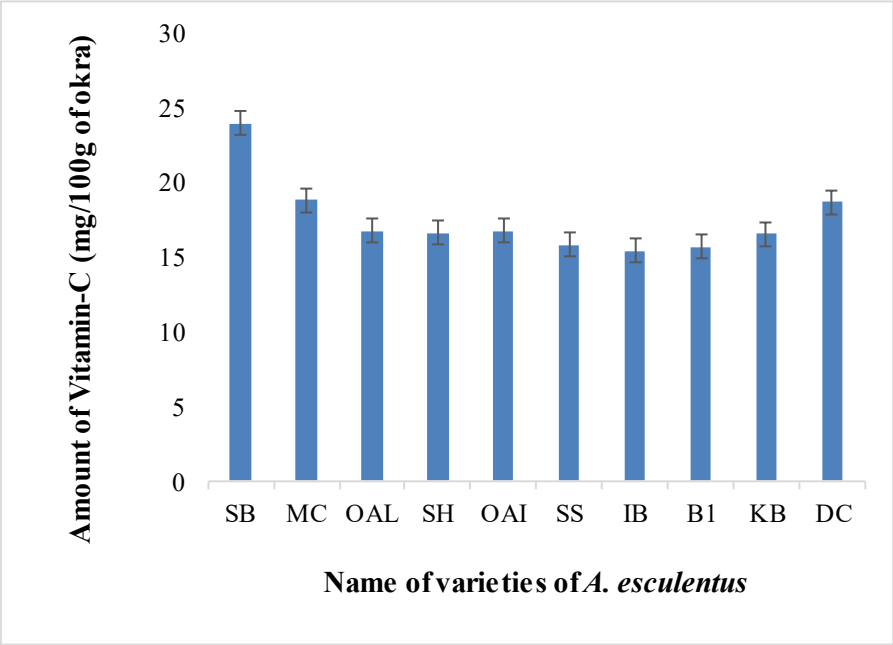

Figure 4.17: Vitamin-C contents of 10 varieties of *A. esculentus*.

4.3.3.11 Mineral content of *A. esculentus* premature pods

Minerals are inorganic elements existing in the body and in food as organic and inorganic combination.

The amount of Calcium (Ca), Potassium (K) and Iron (Fe) present in 10 varieties of *A. esculentus* premature pods are shown in Table 4.12, Figure 4.18, 4.19 and 4.20.

In the present study, the highest amount of calcium was present in Shamol Bangla (SB) i.e 85.61 (mg/100g) of *A. esculentus* premature pods. The 10 varieties premature pods calcium content value ranged from 69.38 to 85.61(mg/100g) which is within the range value than other reported values. Approximately, the edible portion of the okra fruit calcium contained 84.0 g/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978). The compound of okra leaf per 100 g edible portion calcium is 90 mg/100g reported by (Benchasri, 2012a; VarmuDy, 2011; Gopalan *et al*., 2007). Ahiakpa *et al*. (2014) studied that the okra calcium content value varied from 10.12±1.52 mg/kg to 96.21±14.43mg/m. The value of the calcium content were found 25 mg/100g reported by (Dilruba *et al*., 2009; Arapitsas, 2008; Mays *et al*., 2007; Gopalan *et al*., 2007; Owolarafe & Shotonde, 2004; Lamont, 1999). Gemede *et al*. (2016) studied that the calcium value varied from 111.11– 311.95 mg/100g. The calcium content value was

82 mg /100g (USFDA data base report). Sindhu & Puri, (2016) reported that the calcium value was 75 mg/100g. In another report of Nwachukwu *et al*., 2014; Idris *et al*., 2009, the leaf calcium content was 57.03±0.12 mg/100g. Roy *et al*. (2014) investigated that the calcium content was 81 mg/100g. The present value range was found to be most consistent and supported with the other reported values and Shamol Bangla could be used as a good source for calcium.

In the present work, the highest amount of potassium was present in Mahira Cross (MC) i.e 286.60 (mg/100g) of *A. esculentus* premature pods. The 10 varieties premature pods potassium content value ranged from 223.16 to 286.60 (mg/100g) which is within the range value than other reported values. Approximately, the edible portion of the okra fruit potassium contained 20 mg/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978**).** Gemede *et al*. (2016) reported that the potassium value ranged from 122.59–318.20 mg/100g. The potassium content value was 299 mg/100g (USFDA data base report). Sindhu & Puri, (2016) reported that the potassium value was 299 mg/100g. Roy *et al*. (2014) reported that the potassium content was 303 mg/100g. The present value range was found to be most consistent and supported with the other reported values and Mahira Cross (MC) could be used as a good source for potassium.

In the present investigation, the highest amount of iron was present in Orka Anamika Local (OAL) i.e 0.98 (mg/100g) of *A. esculentus* premature pods. The 10 varieties premature pods iron content value varied from 0.62 to 0.98 (mg/100g) which is within the range value than other reported values. Approximately, the edible portion of the fruit iron contained 1.20 mg/100g (Kolawole *et al*., 2011; Saifullah & Rabbani, 2009; Kahlon *et al*., 2007; Ndunguru & Rajabu, 2004; El-Nahry *et al*., 1978). The composition of okra leaf per 100 g edible portion iron is 0.70 mg/100g reported by (Benchasri, 2012a; VarmuDy, 2011; Gopalan *et al*., 2007). Gemede *et al*. (2016) studied that the iron value extended from 18.30–36.68 mg/100g. The iron content value was 0.62 mg/100g (USFDA data base report). In another report of Nwachukwu *et al*., 2014; Idris *et al*., 2009, the leaf iron content was 20.78±0.15 mg/100g. Roy *et al*. (2014) reported that the iron content were 0.8 mg/100g. The present value range was found to be most consistent and supported with the other reported values and Orka Anamika Local could be used as a good source for iron.

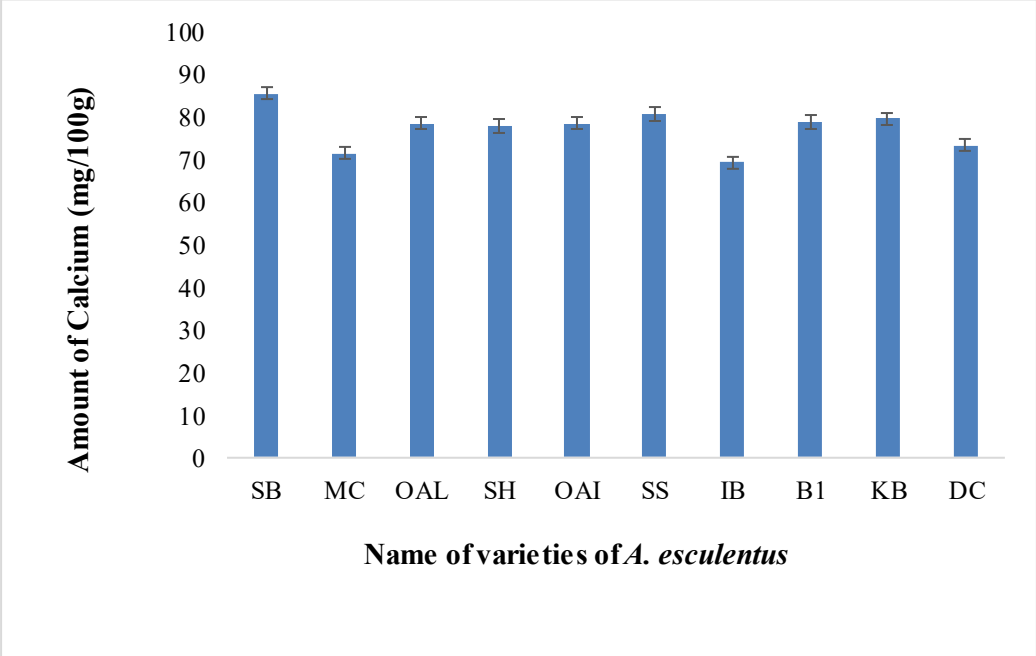

Figure 4.18: Calcium contents of 10 varieties of *A. esculentus*.

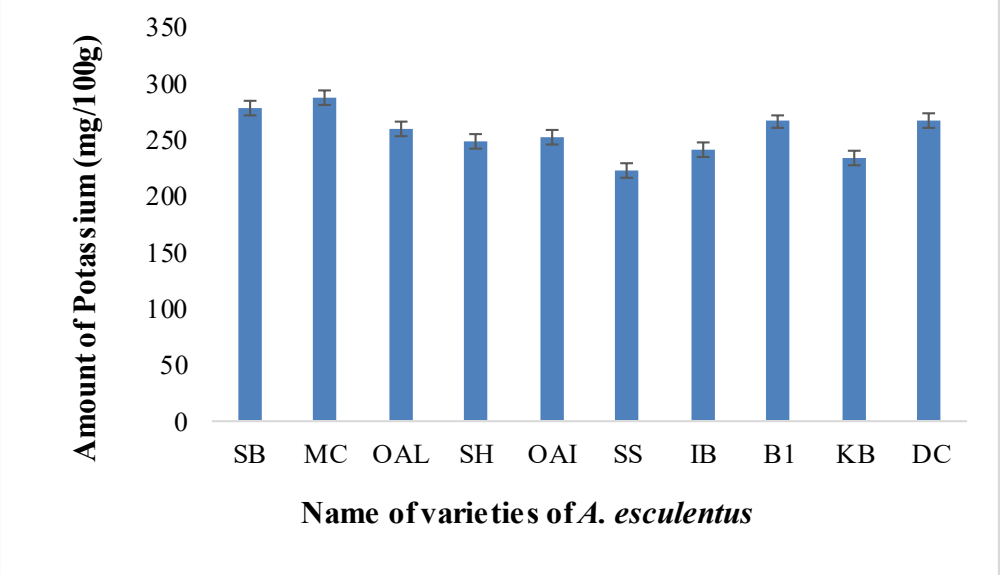

Figure 4.19: Potassium contents of 10 varieties of *A. esculentus*.

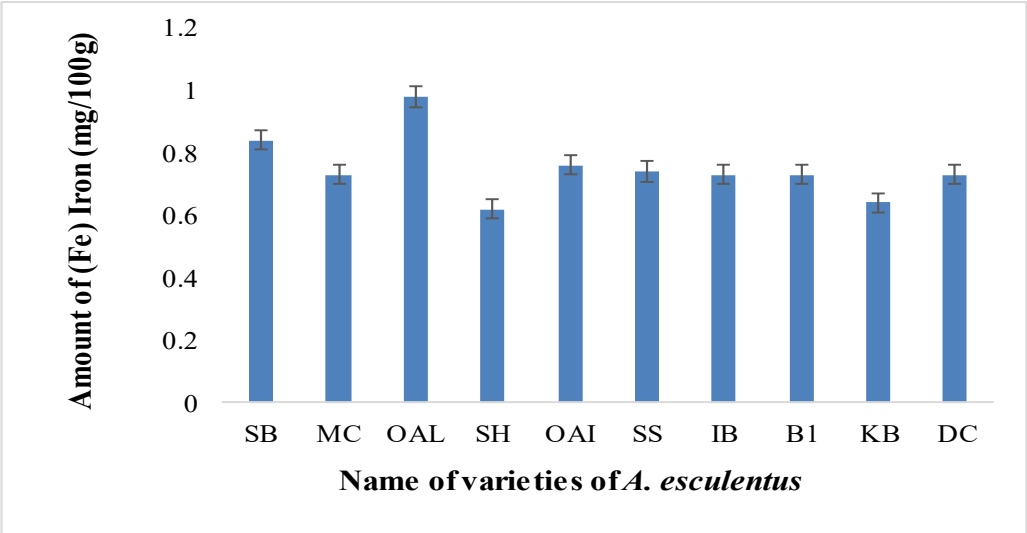

Figure 4.20: Iron contents of 10 varieties of *A. esculentus*.

From the Table 4.12, it is shown that the different types of minerals are present in 10 varieties premature pods of *A. esculentus*. The values (mg/100g) of mineral content (Ca, K and Fe) were varied with the variety of Okra. The highest total amount of minerals (Ca-, K- and Fe-) showed in Shamol Bangla (SB) variety i.e 364.58 (mg/100g) and Lowest total amount of minerals showed in Iron Bhendi (IB) variety i.e 311.46 (mg/100g) among the 10 varieties premature pods of *A. esculentus* and the total amount of mineral content of other 08 varieties were 358.85, 338.94, 326.69, 325.67, 312.52, 345.73, 313.35 and 341.41 (mg per 100g of premature okra pod) for MC, OAL, SH, OAI, SS, B-1, KB and DC respectively. Therefore, the Shamol Bangla can be selected as a good source for higher minerals content among the 10 varieties of okra.

Table 4.12: Biochemical and Nutritional content of 10 varieties of *A. esculentus* premature pods.

Variety	g% (approx.)						mg /100g (approx.)			
	Carbohydrate	Protein	Lipid	Total soluble sugar	Reducing sugar	Sucrose/ Non-reducing sugar	Vitamin-C	Minerals		
								Ca (Calcium)	K (Potassium)	Fe (Iron)
SB	7.42	2.15	0.09	1.15	0.7	0.45	23.95	85.61	278.13	0.84
MC	6.22	1.97	0.05	0.77	0.48	0.29	18.80	71.52	286.60	0.73
OAL	7.13	1.58	0.06	0.98	0.74	0.24	16.72	78.53	259.43	0.98
SH	6.97	1.10	0.08	0.73	0.34	0.39	16.64	77.92	248.15	0.62
OAI	6.52	1.93	0.06	0.85	0.62	0.23	16.75	78.49	252.44	0.76
SS	6.95	1.57	0.11	0.88	0.60	0.28	15.78	80.62	223.16	0.74
IB	6.12	0.97	0.07	0.86	0.61	0.25	15.42	69.38	241.35	0.73
B1	6.91	1.55	0.06	1.11	0.78	0.33	15.65	78.87	266.13	0.73
KB	6.39	0.95	0.09	0.98	0.71	0.27	16.52	79.57	233.14	0.64
DC	7.16	1.53	0.06	1.28	0.81	0.47	18.69	73.45	267.23	0.73

NB: approx.= approximately.

4.4 Summary

Okra is a highly nutritive and medicinally important vegetable crop consumed worldwide. But the knowledge about the biochemical and nutritional, phytochemical as well as medicinal composition of different varieties of okra cultivated in Bangladesh is very limited. This study was attempted to compare the above properties of cultivated 10 varieties of okra at the experimental garden of Plant molecular Biotechnology Laboratory, Rajshahi University Campus, Bangladesh. Therefore, it is necessary to determine and identify the nutrient rich (higher nutrient content) variety among the cultivated 10 varieties of okra.

Premature pods were extracted using methanol as solvent to employ for different methods to completion of the study. Qualitative and quantitative analyses of extracted phytochemicals were done using standard procedures. *In vitro* antioxidant activities of extracted phytochemicals were assayed by employing several methods. Lethality bioassay of the extracts was performed using standard procedures. Several *in vitro* assay models were employed to investigate the medicinal (antibacterial and antidiabetic) properties of the extracts. Another way, okra premature pod extracts were prepared using water as solvent. Different biochemical and nutritional analyses of aqueous extracts were performed using standard procedures.

Analyses of phytochemical (qualitative and quantitative), antioxidants, medicinal, biochemical and nutritional values were performed. Qualitative phytochemical analysis of pods methanolic crude extracts of 10 varieties of this plant successfully showed the presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins. In quantitative analysis, out of twenty seven (27) parameters, Shamol Bangla (SB) was found to be the highest value in fifteen (15) parameters such as: Total phenol (11.37±0.39mg GAE/g), total flavonoid (9.88±0.09mg CAT/g), total flavonol (5.75±0.09 mg QU/g), antioxidants: IC_{50} (µg/ml): 40.32±2.91(DPPH), 53.12±2.1(ABTS); TAC highest (1.285±0.013), FRAP highest (1.293±0.019), ash 10.647%, carbohydrate 7.42%, protein 2.15% (g%), vitamin C 23.95, minerals: Ca 85.61 (mg per 100g), antibacterial activity (zone of inhibition 18 mm for 900 µg/disc), *in vitro* α-amylase inhibition 69.75% and α-glucosidase inhibition activity 73.48%.

The above results suggested that SB is nutrient rich (higher nutrient content) variety among the selected varieties.

CHAPTER FIVE
MUTATION BREEDING

5.1 Introduction

To recover the low yield, develop resistance against disease condition and generate nutrient rich new variety for spreading in wide range to the local and commercial areas, it is necessary to apply mutation breeding technology.

Okra yellow vein mosaic virus is the most devastating disease, which causes colossal losses in the crop by affecting the quality and yield of the fruits. Attempts had been made by several workers to decrease the disease through the use of pesticides (Sastry & Singh, 1973, 1975; Chakraborty & Mukhopadhyay, 1977; Khan & Mukhopadhyay, 1985a; Ramachandran & Summanwar, 1986; Bhagat *et al*., 1997), resistant screening (Arora *et al*., 1992; Sharma *et al*., 1993; Singh & Gupta, 1991; Bora *et al*., 1992; Nath & Saikia, 1992) and other cultural control techniques (Khan & Mukhopadhyay, 1985a,b; Singh & Singh, 1986; Singh *et al*., 1989). But satisfactory control measure has not been accomplished and the disease continues to be a danger to okra cultivation. Moreover, Okra (*Abelmoschus esculentus*) is nutritionally and medicinally important vegetables in worldwide but the knowledge about the biochemical, nutritional composition, phytochemical and medicinal values of different varieties of okra cultivated in Bangladesh is very limited. Therefore, emphasis is needed on breeding to develop YVMV resistant nutrient rich variety.

For mutation breeding technology parent material is a prerequisite. Based on the field performance evaluation in previous study (Chapter 3, Section 3.4) SB was found to be high yielding and resistant against YVMV. Again, from the comparative study of biochemical, nutritional, phytochemical and as well as medicinal properties of cultivated varieties (Chapter 4, Section 4.4), SB was found the nutrient rich (higher nutrient content) variety. Therefore, SB was identified as the best variety among the cultivated 10 varieties and used successively as the parent materials for mutation breeding program.

In this study, genetic improvement of nutrient rich variety of okra through induced mutation technology has been attempted for YVMV resistant new okra variety.

5.1.1 Mutation

A sudden and heritable change in a character of an organism is called mutation. Plasmagene mutations can easily be detected due to their cytoplasmic inheritance pattern. In crop species, many chromosomal mutations (especially those arising due to small changes in chromosome structure) would be classified as gene mutations. Gene mutations may themselves arise due to the replacement of one or more bases of a gene by another (base substitution), deletion or addition of one or more bases. The term mutation was first introduced by de Vries in 1900. The first variety developed through mutation was released in 1950 in Sweden; it was the Primax variety of white mustard (*Brassica hirta*). Mutation can be induced by mutagens. Mutagens are of two types such as physical and chemical mutagens. The entire operation from treating the biological materials with a mutagen to the isolation of useful mutants is termed as mutation breeding. A mutant is an individual, which shows the mutant phenotype.

5.1.2 Induced Mutations

When mutations are produced in response to a treatment with certain chemical or physical agents, they are called induced mutations. The frequencies of induced mutations are much higher than spontaneous mutations. Generally, induced mutations are comparable to the spontaneous mutations in their effects and the variability produced. Mutation induction rarely produces a new allele, which is not already known to occur spontaneously. Mutation breeding programmes are based on induced mutations only mainly because of their high frequency, which makes their exploitation feasible.

5.1.3 Mutagen Sodium Azide (NaN_3)

Sodium azide is the inorganic compound with the formula NaN_3. This colourless salt is the gas-forming element in many vehicle airbag systems. It is utilized for the preparation of other azide compounds. It is an ionic substance, is highly soluble in water, and is very acutely toxic (Jobelius & Scharff, 2000).

In agriculture sector it is applied as a mutagen for crop selection of plants such as rice (Awan *et al*., 2000), barley (Cheng & Gao, 1988) or oats (Rines, 1985).

Agents that induced mutations are called mutagen. Mutagens are of the following two types: (1) physical and (2) chemical mutagen. In this study, Sodium Azide (NaN_3) was used as a chemical mutagen.

5.1.4 Mutation Breeding

The entire operation from treating the biological materials with a mutagen to the isolation of useful mutants is termed as *mutation breeding*. A *mutant* is an individual, which shows the mutant phenotype. Mutation breeding hopes to utilize the useful mutations, which occurs in very low frequencies, viz., Ca. 0.1% of the total mutations. The various steps in mutation breeding are as follows: (1) defining the objective, (2) selection of the variety, (3) decision of the plant part to be treated, (4) selection of the mutagen and dose, (5) mutagen treatment, and (6) handling of M_1 and subsequent generations. Therefore, this study observed in six seasons in different years from the generation M1 to M6 generations.

From 1930 to 2014 greater than three thousand two hundred mutagenic plant types were released (Wikipedia; Schouten & Jacobsen, 2007) which have been derived either as direct mutants (70%) or from their progeny (30%) (Maluszynski et al., 2000). Crop plants account for 75% of released mutagenic species with the remaining 25% ornamentals or decorative flora (Ahloowalia *et al*., 2004).

Notable mutant varieties of Bangladesh are Binasail, Iratom-24 and Binadhan-6 rice mutants; Binamoog-5 mung bean mutant variety (Kharkwal & Shu, 2009).

5.2 Materials and Methods

The materials and methods were used by the following process for the 1st generation Mutant (M1) to 6th generation Mutant (M6) of Shamol Bangla okra variety. Selection and screening of the variety were followed by the standard procedure (Singh, 2000). The generations are described in details separately for mutation breeding program.

5.2.1.1 Preparation of healthy seeds

Healthy mature (ripen) fruits of Shamol Bangla (F1 gen. parent) were harvested (800-1000 g) from Biochemistry Research Garden at the Rajshahi University Campus. Seeds separated from fruits were dried, weighed and healthy seeds were stored in air-tight containers at cool and dry place for the mutation breeding program.

5.2.1.2 Preparation of phosphate buffer solution

Potassium Phosphate (KH_2PO_4) 13.6 g buffer dissolve with 100 ml DH_2O for preparing 1M Phosphate buffer (Stock solution). The stock solution was diluted 10 times to obtain 0.1 M Phosphate buffer (pH 3.0)

5.2.1.3 Preparation of NaN_3 solution

Sodium azide (1.3 g) was dissolved in 20 ml DH_2O to prepare 1M stock solution. Five types of mutagenic treatment solution of NaN_3 were prepared:

T1. 1 mM = 20µl of 1M Sol^n of sodium azide +19.80 ml of 0.1M P. buffer
T2. 2 mM = 40µl of 1M Soln of sodium azide +19.60 ml of 0.1M P. buffer
T3. 3 mM = 60µl of 1M Soln of sodium azide +19.40 ml of 0.1M P. buffer
T4. 4 mM = 80µl of 1M Soln of sodium azide +19.20 ml of 0.1M P. buffer
T5. 5 mM =100µl of 1M Soln of sodium azide +19.00 ml of 0.1M P.buffer
It may be mentioned here that the sodium azide sol^n were not used in control.

5.2.1.4 Seed treatment

Seeds were treated with different concentrations of NaN_3 following published protocols (Nilan *et al*., 1973), with some modifications. Seeds were cleaned with distllied water. Seeds were soaked upto 20 h with different concentrations (1, 2, 3, 4 and 5 mM) of Sodium Azide in the conical flasks. The flasks were shaked with 160 rpm upto 4 h. Seeds were washed with distilled water and transferred to the open field for sowing.

5.2.2 Mutant 1 (M1) okra cultivation

Total land area was 32 m x 10 m for the okra cultivation. Land preparation: Each plot size: 3 m × 1 m, Plant space: 30 cm × 40 cm, 45 cm left for irrigation and drainage between two beds. Manures and fertilizers were used as recommended by Bangladesh

Agricultural Research Institute (Mondal *et al*., 2011). No pesticide was applied during the experimental studies. As the mutant [1st generation of Mutant (M1) okra] seeds were sowed with Randomized Complete Block Design (RCBD) with 3 replicas. Plots were tagged by the name of treatment with seed sowing date. One non-treated control was included to the experiment. Irrigation, weeds cleaning, fertilization etc were maintained according to the local agronomic practice.

5.2.2.1 Data collection

Eleven morphological characteristics of treated and non-treated okra were recorded and followed as mentioned in Section 3.2.3.2 (Chapter 3). The flow diagram of materials and methodology (M1 to M6) are shown below in a flowchart.

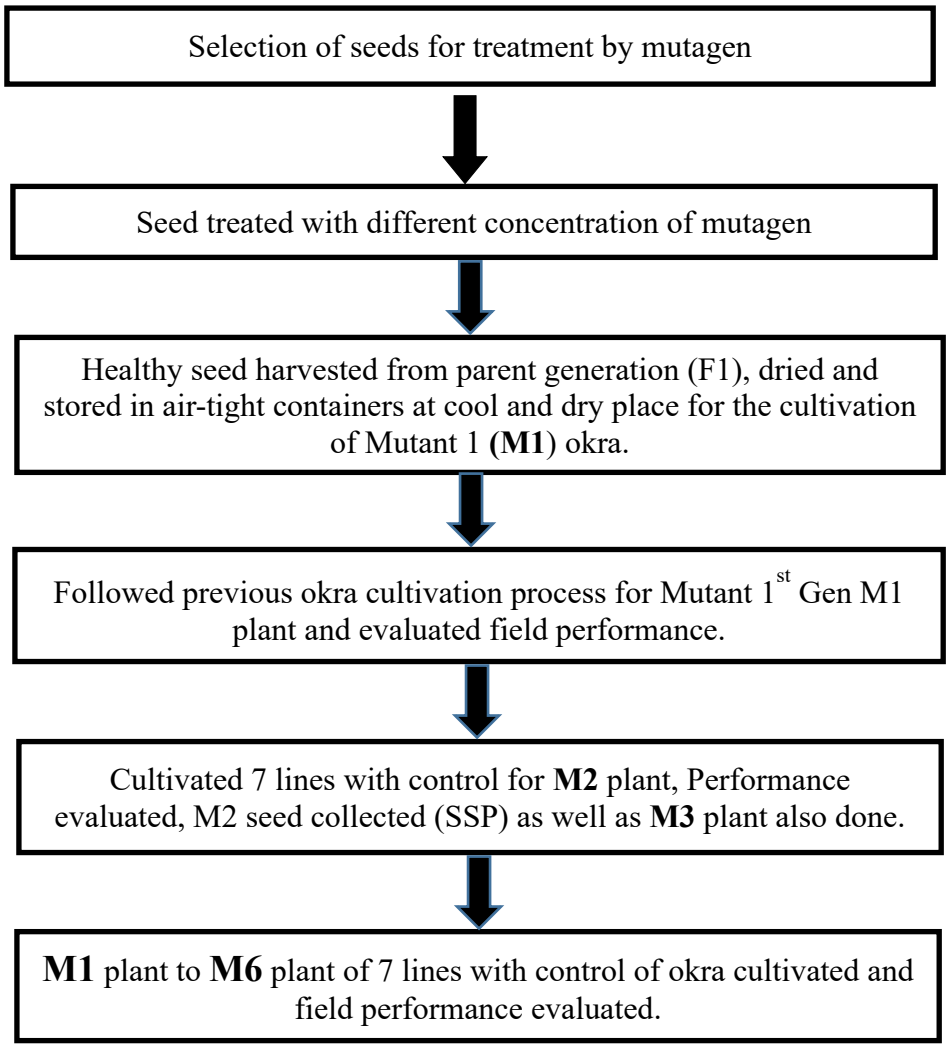

Figure 5.1: The flow diagram of methodology (M1 to M6 gen.)

5.2.3 Mutant 2 (M2) okra plant cultivation

We used single plant selection method or Single Seed Population (SSP) for choosing the mutant plants. Seven M1 plants were selected based on the development of virus (Yellow Vein Mosaic Virus) resistance. Then seeds were collected as M2 seeds (M2 seeds comes from NaN3, 4 mM conc. of M1 plants). Mutant 2 (M2) plants were cultivated in the same garden with the same agromic practice.

Seven virus free plants seeds (M2) were recultivated in next season following the method as mentioned in Section 3.2.3.1 (Chapter Three). These plants were called as Mutant 2 (M2) plants and also termed as indivisual lines (seven lines) such as M2L1, M2L2, M2L3, M2L4, M2L5, M2L6 and M2L7.

Eleven morphological characteristics of seven lines plants were recorded as mentioned in Section 3.2.3.2 (Chapter 3). The screening of Mutant (M2) okra for YVMV resistance was performed during Feb-June 2017.

5.2.4 Mutant 3 (M3) okra plant cultivation

We used bulk collection method to harvest the seeds of each of seven lines from M2 plants which were virus free. The cultivation M3 okra plant was the same as discussed in Section 5.2.3.

These plants were called as Mutant 3 (M3) plants with seven indivisual lines such as M3L1, M3L2, M3L3, M3L4, M3L5, M3L6 and M3L7.

The morphological characteristics were recorded as mentioned in Section 3.2.3.2 (Chapter 3). The screening of Mutant (M3) okra for YVMV resistance was performed during July-Nov, 2017 (Figure 5.1).

5.2.5 Mutant 4 (M4) okra plant cultivation

The seeds of seven lines from M3 plants were virus free. The cultivation M4 okra plant was performed in the same way as discussed in Section 5.2.3.

Healthy M3 seeds were recultivated following the method as mentioned in Section 3.2.3.1. These plants were called as Mutant 4 (M4) plants and also termed as indivisual lines (seven lines) such as M4L1, M4L2, M4L3, M4L4, M4L5, M4L6 and M4L7.

The morphological characteristics of seven lines were recorded as mentioned. The screening of Mutant (M4) okra for YVMV resistance was performed during Feb-June 2018 (Figure 5.1).

5.2.6 Mutant 5 (M5) okra plant cultivation

The seeds of each of seven lines from M4 plants were virus free. The cultivation method of M5 okra plant was the same discussed in Section 5.2.3.

Healthy M4 seeds were recultivated in next season and called as Mutant 5 (M5) plants such as M5L1, M5L2, M5L3, M5L4, M5L5, M5L6 and M5L7.

The morphological characteristics of seven lines were recorded as mentioned in Section 3.2.3.2. The screening of M5 okra for YVMV resistance was performed during July-Nov, 2018 (Figure 5.1).

5.2.7 Mutant 6 (M6) okra plant cultivation

Seeds of seven lines from M5 plants were virus free. The cultivation of M6 okra plant was the same as discussed in Section 5.2.3.

Healthy seeds from M5 were recultivated in next season and called as Mutant 6 (M6) plants such as M6L1, M6L2, M6L3, M6L4, M6L5, M6L6 and M6L7.

The morphological characteristics of seven lines were recorded as mentioned in Section 3.2.3.2. The screening of Mutant (M6) okra for YVMV resistance was performed during Feb-June 2019 (Figure 5.1).

5.3 Results and Discussion

Mutant (M1 to M6) okra was screened for yellow vein mosaic virus resistance. Data were collected on the basis of some morphological characters and virus incidence in all the individual mutant okra. Morphological characters like plant height, branch number per plant, leaves, flowers and fruits per plant, yield performance, virus incidence and other quantitative traits were calculated during the study. In screening of each okra lines, the mean values are shown in Table 5.1 to 5.6. From the tables, there is a significant difference between different characters among the mutant okra genotypes. Detailed description of the first to sixth screening field data are given below:

Figure 5.2: Mutant M1 okra field as mutated by Sodium Azide (NaN_3) mutagen.

Figure 5.3: Field performance of Mutant M2 okra plants.

Figure 5.4: Mutant M3 okra field.

Figure 5.5: Mutant M4 okra field.

Figure 5.6: Mutant M5 okra field.

Figure 5.7: Mutant M6 okra field.

Detailed field performance data of 1st generation M1 to 6th generation M6 okra plant are shown in Table 5.1 to 5.6.

i) Plant height (cm)

In case of plant height, T4 (plant grew from the seed treated with 4 mM mutagen) was found as the tallest (121.92 cm) variety followed by control plant (zero conc. of mutagen) (112.59 cm) T0, T5 (110.32 cm), T3 (99.89 cm) and T2 (96.53). T1 was found as the shortest variety (87.19 cm) among all the mutant okra genotypes (Table 5.1).

In case of Mutant-2 (M2) generation, there were 7 lines among which L5 was found as the tallest (115.82 cm) followed by L2 (110.28 cm), L1 (110.02 cm), Control (109.53 cm) and L3 (109.55 cm). M2L7 line was found as the shortest line (107.68 cm) among the 7 mutant lines okra genotypes. M2L4 (108.66 cm) and M2L6 (108.24 cm) plants were also shorter (Table 5.2).

In case of M3, L7 was found as the tallest (113.52 cm) line followed by L3 (112.61 cm), L5 (112.35 cm), Control (110.37 cm) and L2 (108.39 cm). L6 was found as the shortest line (105.38 cm) among the 7 mutant lines okra genotypes. L4 (106.83 cm) and L1 (106.72 cm) were also shorter (Table 5.3).

In case of fourth generation M4, L6 was found as the tallest (104.26 cm) line followed by M4L5 (101.71 cm), M4L3 (101.49 cm) and Control (101.01 cm). M4L2 was found as the shortest one (95.57 cm) among the 7 mutant lines. M4L1 (97.82 cm), M4L4 (98.52 cm) and M4L7 (98.78 cm) were also shorter (Table 5.4).

In case of M5, L6 was found as the tallest (106.79 cm) line followed by L3 (103.04 cm), L5 (101.35 cm) and Control (101.11 cm). M5L2 was found as the shortest line (96.19 cm) among the 7 mutant lines. M5L1 (98.73 cm), M5L4 (98.73 cm) and M5L7 (99.31 cm) were also shorter (Table 5.5).

In case of M6 plants L6 was found as the tallest (105.51 cm) followed by L3 (104.77 cm), Control (103.58 cm), L5 (102.85 cm) and L7 (101.73 cm). L2 line was found as the shortest line (97.57 cm) among the 7 mutant lines. M6L4 (99.25 cm) and M6L1 (99.69 cm) were also shorter (Table 5.6).

ii) Number of branches per plant

Branching was higher in 4 mM Conc. (2.66) than any other concentrations. Control, 1 mM and 2 mM conc. also showed high branch number (2.33). The lowest branching was found in 3 mM and 5 mM conc (2.00) (Table 5.1).

Branch number was higher in M2L5 line (4.33) than any other lines of mutant okra. Control and M2L3 also showed high branch number. The lowest branches number (3.33) was shown by several lines of okra i,e M2L1, M2L4, M2L6 and M2L7 (Table 5.2).

In M3 generation, branch number was higher in Control (4.00) and M3L3 line than any other lines. M3L5 and M3L7 also demonstrated high branch number. The lowest branches number (3.11) was shown in M3L1. Also lower branch number was noticed in M3L2 and M3L4 (Table 5.3).

Branch number was higher in Control of M4 generation and also in M4L3 line (4.00) than other lines of okra. M4L2 and M4L6 also showed high branch number. The lowest branches number (2.66) was shown in M4L7 (Table 5.4).

Branch number was higher in M5L3 (4.00) than those of other lines of okra. Control, M5L5 and M5L6 also showed high branch number. The lowest branches number (2.66) was found in M5L4 and M5L7 (Table 5.5).

In M6 generation, higher branch number was demonstrated by M6L3 (4.00) than other lines of okra and high branching was also found in M6L6. Control, M6L2, M6L5 and M6L7 also demonstrated high branch number. The lowest branching (2.66) was shown in M6L4 (Table 5.6).

iii) Number of leaves per plant

Number of leaves in each plant was counted and the highest leaves number was seen in 5 mM conc (40.57) followed by Control (39.86), 4 mM (39.58) and 1 mM Conc. (38.74). The lowest number of leaves was counted in 3 mM Conc. (38.19).

In M2 generation, the highest leaves number was seen in M2L5 (42.33) followed by Control, M2L1 and M2L7. The lowest number of leaves (38.00) was counted in M2L2.

Third generation M3, the highest leaves number was seen in M3L7 (40.19) followed by M3L3, M3L2, M3L4 and M3L5. The lowest number of leaves (37.58) was counted in M3L1.

In M4 generation, the highest leaves number was seen in M4L7 (40.05) followed by M4L4, M4L3 and M4L5. The lowest number of leaves (38.25) was found in Control.

The leaves number of M5 generation was counted and the highest number of leaves was found in M5L7 (40.85) followed by M5L4. The lowest number of leaves (37.28) was calculated in M5L3.

In sixth generation, the highest leaves number was found in M6L4 (41.57) followed by M6L7 and M6L5. The lowest number of leaves (38.11) was counted in M6L3.

iv) Number of flowers per plant

Flowers per plant were also counted in the present study and average flowers per plant were found high in Control and 1 mM Conc. (1.66). The lowest flowering (1.00) was demonstrated by several mutagen concentrations i,e 3 mM and 5 mM.

From the study of M2 generation, the highest flowers per plant were found in Control, M2L3 and M2L5 (1.33). The lowest flower number (1.00) was shown by several lines okra i,e M2L1, M2L2, M2L4, M2L6 and M2L7.

In M3 generation, higher number of flowers were demonstrated in Control (1.24) followed by M3L3. The lowest flower number (1.00) was found by several lines okra i,e M3L1, M3L2, M3L4, M3L5, M2L6 and M2L7.

Fourth generation M4, the number of flowers were counted and the highest number of flowers were found in M4L5 (1.83). The lowest flower number (1.00) was found by several lines okra i,e M4L2, M4L4 and M4L6.

In M5 generation, higher number of flowers were found in M5L1 (1.82) followed by M5L3. The lowest flower number (1.00) was noticed by several lines okra i,e M5L2, M5L4 and M5L6.

Flower number was higher (2.66) in several lines of M6 generation i,e M6L3, M6L5 and M6L7 followed by M6L1. The lowest flower number (1.00) was shown by several lines okra i,e M6L2, M6L4 and M6L6.

v) Number of fruits per plant

Fruits number per plant determines the overall yield of that plant. The highest fruit number was recorded in 4 mM Conc. (22.66) followed by Control (22.00), 3 mM Conc. (16.00), 5 mM Conc. (15.00) and 1 mM Conc. (14.33). The lowest fruit number was counted in 2 mM Conc. (12.33).

In M2 generation, Fruits number was higher in Control (26.33) followed by M2L5, M2L1, M2L4 and M2L7. The lowest fruit number (22.00) was found in M2L6.

Fruits number of M3 generation was demonstrated and the highest fruit number was recorded in M3L5 (25.42) followed by Control, M3L1, M3L7 and M3L6. The lowest fruit number (20.82) was counted in M3L2.

Fruits number of M4 generation was higher in M4L5 (26.58) followed by M4L6 (23.84) and M4L7 (23.59). The lowest fruit number (20.74) was counted in M4 L2.

In M5 generation, the highest fruit number was counted in M5L5 (28.16) followed by M5L6 (24.71) and M5L7 (24.67). The lowest fruit number (21.52) was found in M5L2.

Fruits number was higher in M6L5 (28.63) than those of other lines of okra. M6L7 and M6L6 line also showed high fruit number. The lowest fruit number (20.47) was counted in M6L4.

vi) Weight/fruit (g)

Higher weight of fruit means larger size of fruits. 4 mM Conc. recorded the highest fruit weight (32.3 g). Fruit weight was also found high in Control (30.10 g) and 5 mM Conc (22.5 g). On the other hand the lowest weight of 2 mM Conc. fruit was 18.2 g/fruit.

In M2 generation, higher weight of fruit was found in M2L5 (21.22 g). Fruit weight was also found high in M2L1, Control, M2L4 and M2L7. On the other hand, the lowest weight of fruit (18.19 g/fruit) was recorded in M2L6.

Fruit weight of M3 generation was higher in Control (23.18 g). Fruit weight was also found high in M3L4 and M5L5. The lowest weight of fruit (18.34 g/fruit) was found in M3L1.

Fruit weight was higher in M4L1 (23.94 g) than those of other lines of okra. Control, M4L7 and M4L5 also showed high weight of fruit. On the other hand, the lowest weight of fruit (18.88 g) was demonstrated in M4L4.

Fifth generation higher weight of fruit was found in M5L1 (26.18 g). Fruit weight was also found high in Control, M5L7 and M5L5. The lowest weight of fruit (19.03 g) was shown in M5L2.

In M6 generation, higher weight of fruit was recorded in M6L1 (28.48 g). Fruit weight was also found high in M6L5, M6L7 and M6L6. On the other hand, the lowest weight of fruit (19.74 g) was demonstrated in M6L2.

vii) Number of seeds per fruit

As the fruit size of the 4 mM Conc. plant was largest, number of seeds was also found the highest (55.00) followed by Control (47.66), 3 mM Conc. (42.66), 5 mM Conc. (40.16). The lowest number of seeds was counted in 1 mM Conc. (35.00). It may be mentioned here that, this season got selective seven healthy virus free plants (M1) at 4 mM concentration of NaN3 and collected their seeds (M1 seeds comes from 4 mM conc. of M1 plants) for next season screening.

Seed number was higher in Control (76.00) of M2 generation than other lines of okra. M2L2, M2L4 and M2L5 also demonstrated high seed number. The lowest number of seeds (70.66) was counted in M2L1 and M2L6.

In M3 generation, seed number was higher in M3L3 (73.36). M3L6, M3L5 and Control also showed high seed number. The lowest number of seeds (71.33) was counted in M3L2.

Seed number was higher in M4L5 (73.61) of M4 generation. M4L3, M4L7 and M4L6 also counted high seed number. The lowest number of seeds (70.55) was found in M4L4.

Seed number was higher in M5L5 (72.93) than those of other lines of okra. M5L6 and M5L7 also showed high seed number. The lowest number of seeds (70.73) was found in Control.

In M6 generation, higher seed number was demonstrated by M6L7 (72.49) than other lines of okra and high seed number was also found in M6L5, M6L2, M6L4 and M6L6. The lowest number of seeds (70.04) was counted in Control.

viii) Weight/100 seeds (g)

Weight per 100 seeds was higher in M2L5 line (7.61 g) than any other lines of mutant okra. M2L3, Control, M4L4 and M2L1 also showed high weight per 100 seeds. The lowest weight per 100 seeds (6.95 g) was showed in M2L6 (Table 5.2).

In M3 generation, weight per 100 seeds was higher in M3L5 and M3L3 (7.83 g). M3L7, M3L1 and M3L2 also showed high weight per 100 seeds. The lowest weight per 100 seeds (6.93 g) was found in Control. Seed weight was also lower in M3L4 and M3L6.

Weight per 100 seeds was higher in M4L5 (7.69 g) of M4 generation than other lines of okra. M4L3, M4L1 and M4L7 also showed high weight per 100 seeds. The lowest weight per 100 seeds (6.26 g) was found in Control. Seed weight was also lower in M4L2, M4L4 and M4L6.

Weight per 100 seeds was higher in M5L5 (7.73 g) than those of other lines of okra. M5L3, M5L1 and M5L7 also showed high weight per 100 seeds. The lowest weight per 100 seeds (6.42 g) was showed in Control. Seed weight was also lower in M5L2, M5L4 and M5L6.

In M6 generation, higher weight per 100 seeds was demonstrated by M6L5 (7.99 g) than other lines of okra and high weight per 100 seeds was also found in M6L3 and M6L1. The lowest weight per 100 seeds (5.82 g) was showed in Control. Seed weight was also lower in M6L4, M6L2, M6L6 and M6L7.

ix) YVMV disease incidence (%)

No treatment (T0~T5) was found to be fully resistant against YVMV virus. Virus incidence was very high in treatments. T1 plants were observed highly susceptible to

(61.35%) YVMV infection. Virus incidence was also high in T2 plants (55.53%), T3 plants (48.32%) and T4 plants (43.65%). Only T4 plants showed medium category virus resistant (21.42%). T0 (Control) was observed as the best tolerant plants (virus incidence: 16.66%). It may be noted that the T4 (4 mM conc.) was found to generate most virus tolerant okra population cultivated without any pesticide application under open field condition.

In M2 generation, the results revealed that the selected seven (07) lines of mutant okra plants were resistant to Yellow Vein Mosaic Virus. Control line was observed as the tolerant (virus incidence: 24.64%) variety against YVMV.

The results revealed again that the selected seven (07) lines of 3rd generation mutant (M3) plants were resistant to Yellow Vein Mosaic Virus. Control line was observed as the susceptible (virus incidence: 31.26%) variety against YVMV.

Fourth generation M4, the results revealed again that the selected seven (07) lines of mutants were resistant to Yellow Vein Mosaic Virus. Control line was observed as the susceptible (virus incidence: 39.51%) variety against YVMV.

The results revealed again that the selected seven (07) lines of 5th generation mutant (M5) plants were resistant to Yellow Vein Mosaic Virus. Control line was observed as the tolerant (virus incidence: 26.83%) variety against YVMV.

In M6 generation, the results revealed again that the selected seven (07) lines of mutant plants were resistant to Yellow Vein Mosaic Virus. Control line was observed as the susceptible (virus incidence: 31.59%) variety against YVMV.

x) Yield (g) per plant

It is very much important that the yield per plant depends on the number of fruit (size and shape) and fruit weight. 4 mM Conc of mutagen resulted in the highest yielding variety and yield was 731.91 g/plant. High yield was also observed in Control (662.2 g). The lowest yield was recorded in 2 mM Conc. (224.40 g). Others low yield varieties were also observed in 5 mM Conc. (350 g), 3 mM Conc. (328 g) and 1 mM Conc. (270.2 g).

Yield was higher in M2L5 line (546.10 g/plant) than any other lines of mutant okra. Control also showed high yield. The lowest yield (401.98 g) was found in M2L6. Others low yield varieties were also observed in M2L3, M2L2, M2L7, M2L4 and M2L1.

In M3 generation, yield was higher in M3L5 line (532.29 g/plant) among the 7 lines of mutant okra but it was less than the previous (M2 plant) season. High yield was also observed in M3L7 and M3L3. The lowest yield (435.97 g) was found in M3L2. Others low yield varieties were also observed in M3L1, M3L4 and M3L6. It may be mentioned here that Control line was rejected for its susceptibility against YVMV.

Yield was higher in M4L5 line (588.52 g/plant) of M4 generation than other lines of okra and it was higher than the previous (M3 plant) season. High yield was also observed in M4L1, M4L7 and M4L6. The lowest yield (416.73 g) was recorded in M4L2. Others low yield varieties were also showed in M4L4, M4L3 and Control.

Yield was higher in M5L5 line (612.74 g/plant) than those of other lines of okra and the yield showed higher than the previous (M4 plant) season. High yield was also observed in M5L1, M5L7, M5L6 and Control. The lowest yield (409.55 g) was shown in M5L2. Others low yield varieties were also observed in M5L4 and M5L3.

In M6 generation, higher yield was demonstrated by M6L5 line (693.48 g/plant) and the yield showed higher than the previous (M5 plant) season. High yield was also observed in M6L1, M6L7, M6L6 and Control. The lowest yield (433.17 g) was recorded in M6L2. Others low yield varieties were also noticed in M6L4 and M6L3.

Total pod yield on the area (t ha^{-1})

Four mM Conc was found as the highest concentration of the mutagen to give highest yielding variety and total pod yield was 24.39 t ha^{-1}. High yield was also observed in Control (22.07 t ha^{-1}). The lowest yield (7.48 t ha^{-1}) was showed in 2 mM Conc. Others low yield varieties were also observed in 5 mM Conc. (11.66 t ha^{-1}), 3 mM Conc. (10.93 t ha^{-1}) and 1 mM Conc. (9 t ha^{-1}).

M2L5 line was recorded as the highest yielding variety and yield was 18.20 t ha^{-1}. High yield was also observed in Control (16.82 t ha^{-1}). The lowest yield (13.39 t ha^{-1}) was found in M2L6 Others low yield variety were also observed in M2L3 (13.75 t

ha^{-1}), M2L2 (13.79 t ha^{-1}), M2L7 (14.71 t ha^{-1}), M2L4 (14.71 t ha^{-1}) and M2L1 (15.46 t ha^{-1}).

In M3 generation, Control line was rejected for its susceptibility against YVMV. Yield was higher in M3L5 line (17.74 t ha^{-1}) among the 7 lines of mutant okra and it was less than the previous (M2 plant) season. High yield was also observed in M3L7 (16.04 t ha^{-1}) and M3L3 (15.74 t ha^{-1}). The lowest yield (14.53 t ha^{-1}) was showed in M3L2. Others low yield variety were also observed in M3L1 (14.64 t ha^{-1}), M3L4 (15.04 t ha^{-1}) and M3L6 (15.12 t ha^{-1}).

Yield was higher in M4L5 line 19.61 (t ha^{-1}) of M4 generation than other lines of okra and it was higher than the previous (M3 plant) season. High yield was also observed in M4L1 (18.31 t ha^{-1}), M4L7 (17.69 t ha^{-1}) and M4L6 (16.82 t ha^{-1}). The lowest yield (13.89 t ha^{-1}) was recorded in M4L2. Others low yield variety were also observed in M4L4 (14.24 t ha^{-1}), M4L3 (15.56 t ha^{-1}) and Control (16.29 t ha^{-1}).

Yield was higher in M5L5 line (20.42 t ha^{-1}) than those of other lines of okra and it was higher than the previous (M4 plant) season. High yield was also observed in M5L1 (19.34 t ha^{-1}), M5L7 (18.97 t ha^{-1}), M5L6 (17.81 t ha^{-1}) and Control (17.32 t ha^{-1}). The lowest yield (13.65 t ha^{-1}) was recorded in M5L2. Others low yield variety were also shown in M5L4 (14.61 t ha^{-1}) and M5L3 (15.39 t ha^{-1}).

In M6 generation, higher yield was found in M6L5 line (23.11 t ha^{-1}) than other lines of okra and the yield showed higher than the previous (M5 plant) season. High yield was also observed in M6L1 (20.25 t ha^{-1}), M6L7 (19.25 t ha^{-1}), M6L6 (18.61 t ha^{-1}) and Control (16.74 t ha^{-1}). The lowest yield (14.43 t ha^{-1}) was showed in M6L2 Others low yield variety were also observed in M6L4 (15.09 t ha^{-1}) and M6L3 (16.38 t ha^{-1}).

xi) Biomass (kg) per plant

Total biomass of the plant was measured. 5 mM Conc demonstrated the highest biomass (2.91 kg/plant) followed by 2 mM Conc (2.71 kg) and Control (2.62 kg). Medium category biomass was found in 3 mM Conc. (2.38 kg) and 4 mM Conc. (2.41 kg). The lowest biomass was counted in 1 mM Conc. (2.13 kg).

In M2 generation, total biomass of the plant was measured. M2L5 was recorded the highest biomass (2.92 kg/plant) followed by M2L2, M2L6 and Control. Medium

category biomass was found in M2L3, M2L4 and M2L7. The lowest biomass (2.15 kg) was counted in M2L1.

Total biomass was higher in M3L2 (2.78 kg/plant) of M3 generation. M3L3 and M3L7 also showed high biomass. Medium category biomass was found in M1L1 and M3L5. The lowest biomass (2.28 kg) was shown in M3L6.

Total biomass was higher in M4L1 (2.81 kg/plant) than those of other lines of okra. M4L3, M4L2 and Control also showed high biomass. Medium category biomass was found in M4L5 and M4L7. The lowest biomass (2.06 kg) was shown in M4L6.

In M5 generation, total biomass was higher in M5L3 (2.83 kg/plant) than any other lines. M5L4 and M5L1 also demonstrated high biomass. Medium category biomass was found in M5L5, M5L6 and M5L7. The lowest biomass (2.28 kg) was noticed in Control.

Total biomass was higher in M6L6 (2.93 kg/plant) of M6 generation. M6L5 and M6L2 also showed high biomass. Medium category biomass was found in M6L4, M6L3, Control and M6L7. The lowest biomass (2.13 kg) was shown in M6L1.

Note: 1st generation M1 okra - instead of weight (g) per 100 seeds, emergence (seed to plant) under field condition (days) were calculated: For all concentrations of mutagen (1 mM to 5 mM), emergences of seed to plant were found within 4 days. The control emerged within 3 days (Table 5.1).

Table 5.1: Morphological characteristics of M1 mutant okra plants. Data were recorded at 90th day after seed sowing (July-Nov. 2016).

NaN3 Conc. (mM)	Height (cm) /plant	Emergence under field condition (days)	Branches /plant	Leaves /plant	Flowers /plant	Fruits /plant (a)	Wt(g)/fruit (c)	Seeds /fruit	YVMV infected plants (%)	Yield (g) /plant (axc)	Biomass (kg) /plant
Control	112.59	03	2.33	39.86	1.66	22.00	30.10	47.66	16.66	662.2	2.62
1	87.19	04	2.33	38.74	1.66	14.33	19.3	35.00	61.35	270.2	2.13
2	96.53	04	2.33	38.42	1.33	12.33	18.2	39.00	55.53	224.40	2.71
3	99.89	04	2.00	38.19	1.00	16.00	20.5	42.66	48.32	328.00	2.38
4	121.92	04	2.66	39.58	1.33	22.66	32.3	55.00	21.42	**731.91**	2.41
5	110.32	04	2.00	40.57	1.00	15.00	22.5	40.16	43.65	350.00	2.89

Table 5.2: Morphological characteristics of M2 mutant okra plants. Data were recorded at 90th day after seed sowing (Feb-June 2017).

Plants	**Height (cm) /plant**	**Branches /plant**	**Leaves /plant**	**Flowers /plant**	**Fruits /plant (a)**	**Wt(g)/fruit (c)**	**Seeds /fruit**	**Wt(g) /100 seeds**	**YVMV infected plants (%)**	**Yield (g) /plant (axc)**	**Biomass (kg) /plant**
Control line (Shamol Bangla)	109.53	4.00	39.66	1.33	26.33	19.17	76.00	7.52	24.64	504.82	2.36
Mutant line M2L1	110.02	3.33	39.33	1.0	23.66	19.57	70.66	7.46	-	463.97	2.15
Mutant line M2L2	110.28	3.66	38.00	1.0	22.33	18.47	71.33	7.39	-	413.85	2.62
Mutant line M2L3	109.55	4.00	38.66	1.33	22.33	18.47	72.00	7.54	-	412.75	2.31
Mutant line M2L4	108.66	3.33	38.66	1.0	23.00	19.02	72.11	7.49	-	441.39	2.19
Mutant line M2L5	115.82	4.33	42.33	1.33	25.66	21.22	71.69	7.61	-	**546.10**	2.92
Mutant line M2L6	108.24	3.33	38.66	1.0	22.00	18.19	70.66	6.95	-	401.98	2.41
Mutant line M2L7	107.68	3.33	39.00	1.0	23.00	19.02	71.00	7.17	-	441.39	2.16

Table 5.3: Morphological characteristics of M3 mutant okra plants. Data were recorded at 90th day after seed sowing (July-Nov. 2017).

Plants	**Height (cm) /plant**	**Branches /plant**	**Leaves /plant**	**Flowers /plant**	**Fruits /plant (a)**	**Wt(g)/fruit (c)**	**Seeds /fruit**	**Wt(g) /100 seeds**	**YVMV infected plants (%)**	**Yield (g) /plant (axc)**	**Biomass (kg) /plant**
Control line (Shamol Bangla)	110.37	4.00	37.91	1.24	24.42	23.18	72.00	6.93	31.26	566.05	2.35
Mutant line M3L1	106.72	3.11	37.58	1.0	23.95	18.34	71.37	7.46	-	439.24	2.32
Mutant line M3L2	108.39	3.47	39.46	1.0	20.82	20.94	71.33	7.39	-	435.97	2.78
Mutant line M3L3	112.61	4.00	39.57	1.33	22.82	20.70	73.36	7.83	-	472.47	2.59
Mutant line M3L4	106.83	3.58	39..15	1.0	20.91	21.59	71.62	7.14	-	451.44	2.36
Mutant line M3L5	112.35	3.92	38.59	1.00	25.42	20.94	72.35	7.83	-	**532.29**	2.30
Mutant line M3L6	105.38	3.62	37.94	1.0	23.21	19.55	72.86	7.13	-	453.75	2.28
Mutant line M3L7	113.52	3.81	40.19	1.0	23.39	20.58	73.00	7.58	-	481.37	2.59

Table 5.4: Morphological characteristics of M4 mutant okra plants. Data were recorded at 90th day after seed sowing (Feb-June 2018).

Plants	Height (cm) /plant	Branches /plant	Leaves /plant	Flowers /plant	Fruits /plant (a)	Wt(g)/fruit (c)	Seeds /fruit	Wt(g) /100 seeds	YVMV infected plants (%)	Yield (g) /plant (axc)	Biomass (kg) /plant
Control line (Shamol Bangla)	101.01	4.00	38.25	1.18	21.58	22.65	71.12	6.26	39.51	488.72	2.53
Mutant line M4L1	97.82	3.74	38.39	1.26	22.95	23.94	71.38	7.21	-	549.41	2.81
Mutant line M4L2	95.57	3.83	38.72	1.0	20.74	20.11	70.57	6.73	-	416.73	2.59
Mutant line M4L3	101.49	4.00	39.66	1.72	22.03	21.19	72.68	7.58	-	466.94	2.64
Mutant line M4L4	98.52	3.66	39.81	1.0	22.64	18.88	70.55	6.83	-	427.33	2.73
Mutant line M4L5	102.71	3.66	39.17	1.83	26.58	22.14	73.61	7.69	-	**588.52**	2.18
Mutant line M4L6	104.26	3.82	38.28	1.0	23.84	21.18	72.17	6.88	-	504.86	2.06
Mutant line M4L7	98.78	2.66	40.05	1.15	23.59	22.49	72.32	7.17	-	530.74	2.11

Table 5.5: Morphological characteristics of M5 mutant okra plants. Data were recorded at 90th day after seed sowing (July-Nov. 2018).

Plants	Height (cm) /plant	Branches /plant	Leaves /plant	Flowers /plant	Fruits /plant (a)	Wt(g)/fruit (c)	Seeds /fruit	Wt(g) /100 seeds	YVMV infected plants (%)	Yield (g) /plant (axc)	Biomass (kg) /plant
Control line (Shamol Bangla)	101.11	3.66	37.81	1.33	22.38	25.89	70.73	6.42	26.83	519.81	2.28
Mutant line M5L1	98.73	3.38	38.88	1.82	22.16	26.18	71.64	7.19	-	580.37	2.66
Mutant line M5L2	96.19	3.59	39.15	1.0	21.52	19.03	71.29	6.78	-	409.55	2.29
Mutant line M5L3	103.04	4.00	37.28	1.66	22.94	20.13	71.55	7.61	-	461.92	2.83
Mutant line M5L4	98.73	2.66	40.14	1.0	21.88	20.03	70.81	6.81	-	438.41	2.78
Mutant line M5L5	101.35	3.66	39.59	1.28	28.16	21.75	72.93	7.73	-	**612.74**	2.51
Mutant line M5L6	106.79	3.66	38.61	1.0	24.71	21.63	72.68	6.91	-	534.48	2.42
Mutant line M5L7	99.31	2.66	40.85	1.55	24.67	23.08	72.31	7.14	-	569.39	2.31

Table 5.6: Morphological characteristics of M6 mutant okra plants. Data were recorded at 90th day after seed sowing (Feb-June 2019).

Plants	Height (cm) /plant	Branches /plant	Leaves /plant	Flowers /plant	Fruits /plant (a)	Wt(g)/fruit (c)	Seeds /fruit	Wt(g) /100 seeds	YVMV infected plants (%)	Yield (g) /plant (axc)	Biomass (kg) /plant
Control line (Shamol Bangla)	103.58	3.66	38.57	1.66	21.86	22.98	70.04	5.82	31.59	502.38	2.54
Mutant line M6L1	99.69	3.47	39.32	1.66	21.33	28.48	70.72	7.39	-	607.59	2.13
Mutant line M6L2	97.57	3.66	38.73	1.0	21.94	19.74	71.55	6.26	-	433.17	2.79
Mutant line M6L3	104.77	4.00	38.11	2.66	22.58	21.76	70.81	7.88	-	491.53	2.35
Mutant line M6L4	99.25	2.66	41..57	1.0	20.47	22.11	71.53	6.05	-	452.77	2.16
Mutant line M6L5	102.85	3.66	40.21	2.66	28.63	24.22	71.68	7.99	-	**693.48**	2.81
Mutant line M6L6	105.51	3.84	39.83	1.0	23.92	23.34	71.17	6.49	-	558.36	2.93
Mutant line M6L7	101.73	3.66	40.79	2.66	24.15	23.91	72.49	6.86	-	577.62	2.55

In the study, it was observed from the data in Table 5.1 that the 4 mM Conc of the mutagen resulted in medium virus tolerance (21.42%). As the yield per plant depends on the number of fruit (size and shape) and fruit weight, 4 mM Conc demonstrated the highest yield (731.91 g/plant). Control was observed as the best tolerant variety (virus incidence: 16.66%) but the yield was observed in the second highest (662.2 g). The lowest yield was recorded in 2 mM Conc. (224.40 g). Others low yield variety were also observed in 5 mM Conc. (350 g), 3 mM Conc. (328 g) and 1 mM Conc. (270.2 g). This result revealed some consistency in comparison to other reported results.

Al-Qurainy (2009) reported that the *Eruca sativa* seedlings developed from treated seeds with sodium azide at concentration ranged (1x 10^{-3} to 5x10^{-3} M) showed wide variation in plant growth (plant height, leaf area, fresh and dry weight) upto 60 days after sowing. Among various concentrations of sodium azide, 3 mM was found incredibly powerful in all morphometric and yield traits after 60 days of sowing in comparison to untreated plants. General chlorophyll content reduced (chlorophyll content may be the mutation at various loci of genome at various concentration of sodium azide) as compared to control plants and low content material of chlorophyll was determined in those plants which advanced from treated seeds at 5 mM concentration of sodium azide. Their results had been contradictory to results of Cortes (1973), Niknejad (1976), who labored with concentrations (1 and 4x10 M) and 5 to l00 x 10 M and determined that the lowest concentration induced a better frequency of chlorophyll-deficient mutations. They advise that particular dose of sodium azide, (that is 3 mM for 2 h treatment) can be used to create genetic variability in *Eruca sativa*, which would be the idea for quality improvement in this species.

Sable *et al*. (2018) reported on germination percentage and morphological growth in two types of okra at the different concentration of sodium azide. They found that the incresing dose of sodium azide was appeared adverse impact on germination percentage (%) as well as morphological characters of two different varieties of okra. The highest fresh weight of okra plant in control treatment T0 (0.00% of sodium azide) was about (3.66 g) in Parbhani Kranti and (2.11 g) in Arka Anamika followed to (2.43 g). They concluded that sodium azide at (0.04% of sodium azide) low concentration appeared to be the better effective treatment for inducing variality in

okra varieties such as Parbhani Kranti and Arka Anamika as compare with other concentrations of sodium azide (0.08 % to 0.24 %).

Boonsirichai *et al*. (2009) reported on the genetics of the radiation prompted yellow vein mosaic disease (YVMD) resistance mutation in okra in Thailand. They try to increase a DNA marker for YVMD-resistance via gamma irradiation of the Okura variety form of okra. Analysis of F1 and F2 progeny found out the semi-dominant nature of the resistance which appeared to be because of a single-locus mutation. MFLP fingerprintings of the F2 and the BC1F1 populations revealed a DNA fragment that is doubtlessly related to the mutation, the visible assessment of YVMD, a PCR technique was developed for the assay of the presence of YVMD virus in leaf tissues.

From the above study it was clear that the dose of sodium azide can be used to create genetic variability for quality improvement of okra. Therefore, the 4 mM conc. dose can be selected for the production of best quality okra through induced mutaion.

In respect of yield, from the analysis of Mutant 2nd Gen (M2) to Mutant 6th Gen (M6) okra, it was observed that the highest yield showed in the line M6L5 693.48 g/plant (23.11 t ha^{-1}) and was found resistant to YVMV (plants were screened under open field condition without any pesticide application) while the control highest was 566.05 g/plant (18.86 t ha^{-1}) with YVMV. Also the M6L5 line yield was showed the highest yield 23.11 (t ha^{-1}) compared to the previous studied field performance (chapter-3) highest yield 17.35 t ha^{-1} (520.78 g/plant) of Shamol Bangla (Parent). This study value (23.11 t ha^{-1}) is a little bit higher than any other reported (chapter-3) data. So, mutant okra lines might be the right choice for cultivation. Therefore, the YVMV resistant nutrient rich mutant M6L5 line can be selected to get more yield (highest yield) for widely cultivation among the 7 lines of nutrient rich mutant okra.

5.4 Summary

For mutation breeding technology right selection parent material is a prerequisite. In previous study (Chapter 3, Section 3.4) based on the field performance evaluation, Shamol Bangla (SB) showed the high yielding and was found to be resistant against YVMV. Another way, in previous study (Chapter 4, Section 4.4) based on the evaluation of comparative study of biochemical, nutritional, phytochemical and as

well as medicinal properties of cultivated varieties, Shamol Bangla (SB) was found the nutrient rich (higher nutrient content) variety. Therefore, Shamol Bangla was identified as the best variety among the cultivated 10 varieties of okra and it was used as the parent materials for mutation breeding program. In this study, genetic improvement of nutrient rich variety of okra through induced mutation technology has been attempted for YVMV resistant new okra variety.

Screened nutrient rich variety was used for mutation-breeding to develop YVMV resistant mutant okra lines. Seeds were treated with sodium azide (NaN_3) at concentrations of 1, 2, 3, 4 and 5 mM for chemical mutagenesis, and sown under open field condition without any pesticide application. The subsequent generations with YVMV resistance were screened starting from mutant generation-1 (M1) to mutant generation-6 (M6).

Hence, SB was selected for mutation breeding to develop YVMV resistant new okra variety. NaN_3 at 4 mM concentration showed the highest yield 731.91 g/plant (24.39 t ha^{-1}). Seven healthy virus free plants (M1) were selected and their seeds (M1 seeds) were collected for next season screening. M2 plants were grown under similar condition and Single Seed Population (SSP) method was used to screen the resistant lines. The results revealed that the selected lines (M2-M6) were resistant to YVMV. Among M2 lines, the yield of M2L5 was the highest 546.10 g/plant (18.20 t ha^{-1}). Among M3 lines, the yield of M3L5 was the highest 532.29 g/plant (17.74 t ha^{-1}) but less than the M2 plants. On the other hand, the yield M4L5 was the highest 588.52 g/plant (19.61 t ha^{-1}), which is higher than M3 plants. On the other hand, the yield of M5L5 was the highest 612.74 g/plant (20.42 t ha^{-1}) and was higher than M4 plants. In M6 mutant plants, the yield of M6L5 was the highest 693.48 g/plant (23.11 t ha^{-1}) and was higher than the M5 plants.

From the above results, it may be concluded that the line 5 always have showed the best line with highest yield (M5L5 i.e 612.74 g/plant and M6L5 i.e 693.48 g/plant) that had resistance against YVMV among the mutant 7 lines. Therefore, the line 5 was selected for further analysis.

CHAPTER SIX
MOLECULAR CHARACTERIZATION ADVANCED LINES OF MUTANT OKRA USING MOLECULAR MARKERS

6.1 Introduction

Molecular characterization study of mutant okra advanced lines will provide an opportunity for plant breeders to develop new and improved cultivars with YVMV resistant characteristics.

For the fulfillment of any crop improvement program the availability of genetic diversity is a needful (Haq *et al*., 2013). Relationship among okra germplasm and their genetic variability investigation may additionally play important function in plant breeding program associated with the biotic and abiotic stress tolerance (Gulsen *et al*., 2007). In various crop improvement and plant breeding programs and also in the evolutionary biology characterization and quantification of genetic diversity has long been a major goal. For a rational use of plant genetic resources, genetic diversity information within closely related crops is very important (Ramanatha Rao & Hodgkin, 2002).

Genetic variability can be created by different mutation methods among individuals or it can naturally exist in the gene pool (Haq *et al*., 2013). Molecular markers are now considered an efficient and powerful tool in the study of genetic variability study among different closely related groups and in the elucidation of genetic relationships within these species (Chakravarthi & Naravaneni, 2006). In the field of molecular genetics use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most outstanding developments. To protect and use these plant genetic materials effectively, it is essential to develop markers that not only differentiate individuals and accessions, but also indicate the inherent diversity and relationships among collection holdings (Ibrahim *et al*., 2010).

Different types of molecular markers having variation in their principles and methodologies are available such as AFLP, SSR, RFLP and RAPD for study the

genetic diversity (Kaur *et al*., 2013). Among all the several DNA based techniques, Random Amplified Polymorphic DNA (RAPD) is quite simple, less technology intensive, cheaper and it does not require any pre-sequencing for designing primers (Haq *et al*., 2013; Kaur *et al*., 2013). RAPD markers have been applied as a tool to estimate genetic diversity in a number of vegetable crops species like Triticum, Cotton, Okra, Radish, Capsicum, Onion and many other crops (Kaur *et al*., 2013). It has become very essential to identify individual varieties due to the number of genetically related varieties released by the way of breeders has made morphological identification more hard and the DNA fingerprints end up the genetic identity of a genotype. The goal of the study was to gain information about genetic diversity of mutant okra at molecular level. This information will be useful in the breeding programs for the development of new varieties of okra with desired agronomic traits such as virus resistance. This study was undertaken to evaluate the molecular characterization of the YVMV resistant mutant lines by the molecular markers such as RAPD and SSR and finally to identify the YVMV resistant gene in mutant okra lines.

6.1.1 Types of molecular markers

Various types of molecular markers are now being used all over the world to estimate genetic diversity of plants and these markers can be classified as the hybridization-based molecular marker and the polymerase chain reaction (PCR) based molecular marker (Stuber *et al*., 1999). In the hybridization-based molecular marker system, DNA fragments are digested with a restriction endonuclease and are hybridized with the labelled probe (A DNA fragment of known sequence). In PCR based molecular marker system, DNA fragments are amplified in the thermal cycler using specific or arbitrary sequences (primer). Some primers are gene-specific and some are randomly selected. The amplified PCR products are visualized by electrophoresis or autoradiography. After electrophoresis, the products are viewed under the gel documentation system. PCR is extremely sensitive and functions very rapidly. Hybridization-based molecular marker system is time-consuming and complicated in their principle. For this reason, PCR based molecular marker is used very widely in the current years.

New molecular markers are frequently developing besides the use of existing marker systems. Today, no method is ideal for all applications; so it is very important to choose an appropriate marker system for a specific project (Hartl & Jones, 2005). Molecular markers can be divided according to their dominant and co-dominant behaviour. The co-dominant markers can distinguish between homozygotes and heterozygotes. The most frequently used dominant DNA marker for genetic diversity in plants are: Random Amplified Polymorphic DNA, Arbitrarily primed polymerase chain reaction (AP-PCR), Inter-simple sequence repeat (ISSR), Amplified Fragment Length Polymorphisms DNA Amplification fingerprinting, Restriction Fragment Length Polymorphisms (RFLP), Microsatellites (SSR), Sequence characterised amplified regions (SCAR), Cleaved amplified polymorphic sequence (CAPS), Expressed sequence tag (EST)) and Single Nucleotide Polymorphism (SNP) and sequence tagged sites (STS).

Among all the molecular markers Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) Marker are selected for the present study.

RAPD (pronounced 'rapid'), for Random Amplification of Polymorphic DNA, is a type of molecular marker system in which random primers of short length (10 bp) are used to amplify the gDNA. The random primers search over the full genome and bind at some locus of the DNA. The RAPD analysis is described by Williams *et al*. (1990). It is a frequently used molecular marker in genetic diversity studies. Prior knowledge of the DNA sequence is not required for the targeted gene, as the primers will bind somewhere in the sequence, but it is not sure exactly where the primers will bind.

RAPD technique has been used in a number of crops by number of research groups, like, Triticum, Celery, Barley, Papaya, Cotton, Okra, Radish, Capsicum, Onion, Ginger, Watermelon, Tomato etc (Litt & Lutty, 1989).

The standard RAPD technology utilizes short synthetic primers of random sequences as primers to amplify very small quantities of total gDNA at a low annealing temperature by PCR (Tingey, 1990). Generally, agarose gels stained with ethidium bromides are used for the separation of the amplified product. As the length of random primer used in RAPD experiment is smaller in size and also in the amplification condition, they all differ from the standard PCR condition (Kumari &

Thakur, 2014). Generation of RAPD band is primarily because of the homology of the nucleotide sequence of genomic DNA with the primer sequence and it depends on different annealing temperature

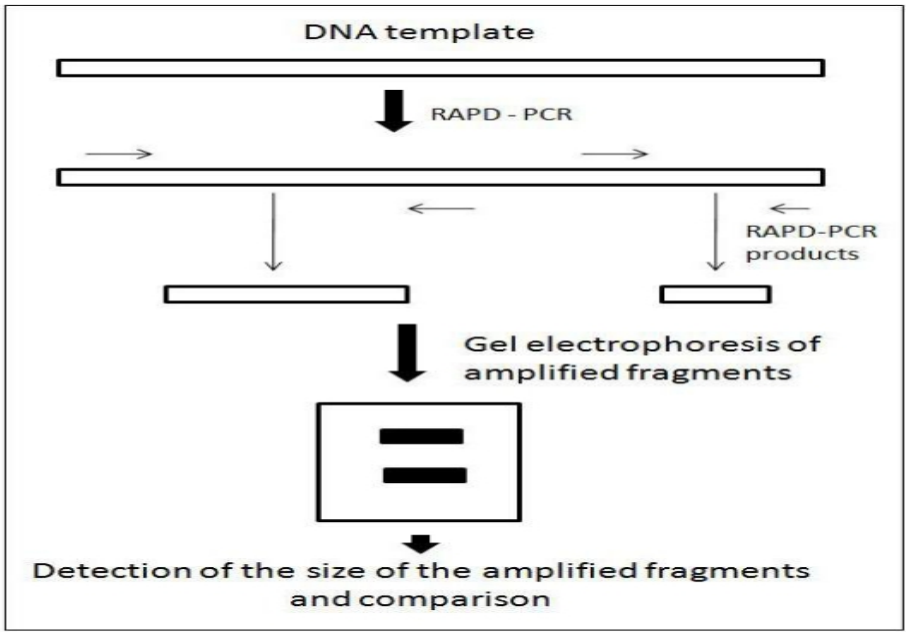

Figure 6.1: Flow diagram of RAPD

RAPD technique has some advantages over the other DNA based molecular marker techniques. The major advantages of RAPD are;

- It is quite a simple and quick process.
- Less technology intensive.
- Cheaper and more available than any other molecular markers.
- It does not require any pre-sequencing for designing primers.
- A small amount of DNA is required.

Considering all the advantages, the RAPD marker is selected for the present study of genetic diversity analysis among different varieties of okra germplasm.

Among all the molecular markers microsatellite or SSR marker has drawn great attention of the scientist in the recent years especially for plant breeding purposes. Microsatellite markers are usually tandemly repeated (2-6) clusters of base sequence distributed throughout the whole genome (Litt & Lutty, 1989). SSR marker has

distinguished itself from another molecular marker as it is highly polymorphic, co-dominant, multialellic DNA marker requiring a very lower amount of genomic DNA.

The main drawback of the SSR marker is the high cost of developing the primers for a specific species. Microsatellites are thought to be generated by the impairing of one strand of DNA during replication of double-stranded DNA or it may also be raised by unequal crossing over during meiosis (Ellegren, 2004).

Microsatellite marker is considered as the most efficient marker of all the molecular marker system but its use is not frequent for all the species due to the high cost and laborious effort to develop microsatellite marker prior to the main experiment. There are different methods to develop microsatellite marker in a specific species. The conventional method for development of microsatellite marker includes searching genomic library using microsatellite motif as a probe. Another method includes searching for a sequence containing microsatellite motif in the database like genebank, EMBL. The second method has been applied in okra and okra related plants like Cotton, Hibiscus and also other plants like Sugarcane, Rice, Wheat, Barly etc (Qureshi *et al*., 2004; Bruna *et al*., 2009; Rota *et al*., 2005).

SSR marker developed for one particular species may be applied to a huge range related species also. Due to the conserved region present in the genome, DNA markers can be transferred across species. Microsatellite marker is first developed for an economically important crop, then the efficiency of this marker to the other related species can be tested. Some economically important crop plants have created enough interest and for this, the cost required for the development of SSR marker for such plants is somehow managed whenever some less economically important crops don't have this facility. For such minor plants the SSR marker developed for related species are tested and this effort is also useful to check the transferability of SSR marker among different genomes. This method is useful for germplasm collection and evaluation (Wang *et al*., 2004). This process depends on the conservation of the priming region of SSR marker within flanking regions to enable amplification.

Transferability of SSR marker across the different species has made this marker system more powerful tool for comparative genetic analysis. Although the development of microsatellite marker is cost-effective and time-consuming, this

marker system has advantages over other markers like RAPD, RFLP, AFLP, on several aspects such as being PCR based, co-dominant marker, highly reproducible, multiallelic, easy and fast assay of genotypes. Microsatellite marker has been strongly applied in the genetic fingerprinting, population genetics analysis, genetic mapping, marker-assisted selection (MAS) and genotype identification of individuals.

Microsatellite DNA loci have been considered as an important source of genetic information for different purposes. To amplify microsatellite loci by PCR, primers are required to develop from the flanking region of the DNA sequence and this region of the DNA is most variable in the genome. Thus the binding sites of primers are not conserved to the distantly related individual and polymorphism will occur (Primmer *et al*., 1996; Moore *et al*., 1991). Keeping all the advantages, disadvantages and application, RAPD and SSR marker were used to study genetic diversity among the 7 advanced lines of mutant okra.

6.2 Materials and Methods

6.2.1 Plant materials

A total of seven advanced lines of mutant okra SB with control were included in this study. Young healthy leaves collected from the mutant M5 plants were used in this experiment as listed in Table 6.1 bellow.

Table 6.1: List of mutant lines (M5) of SB with control used for the experiment.

SL. No.	Name of Lines	Sample collection
1	Mutant 5 Line 1 = M5L1	Young leaves of M5 plants of okra collected from the cultivated garden of Biochemistry, RU.
2	Mutant 5 Line 2 = M5L2	
3	Mutant 5 Line 3 = M5L3	
4	Mutant 5 Line 4 = M5L4	
5	Mutant 5 Line 5 = M5L5	
6	Mutant 5 Line 6 = M5L6	
7	Mutant 5 Line 7 = M5L7	
8	Control -Shamol Bangla-C.	

6.2.2 Methods:

The flow diagram of materials and methods are shown below.

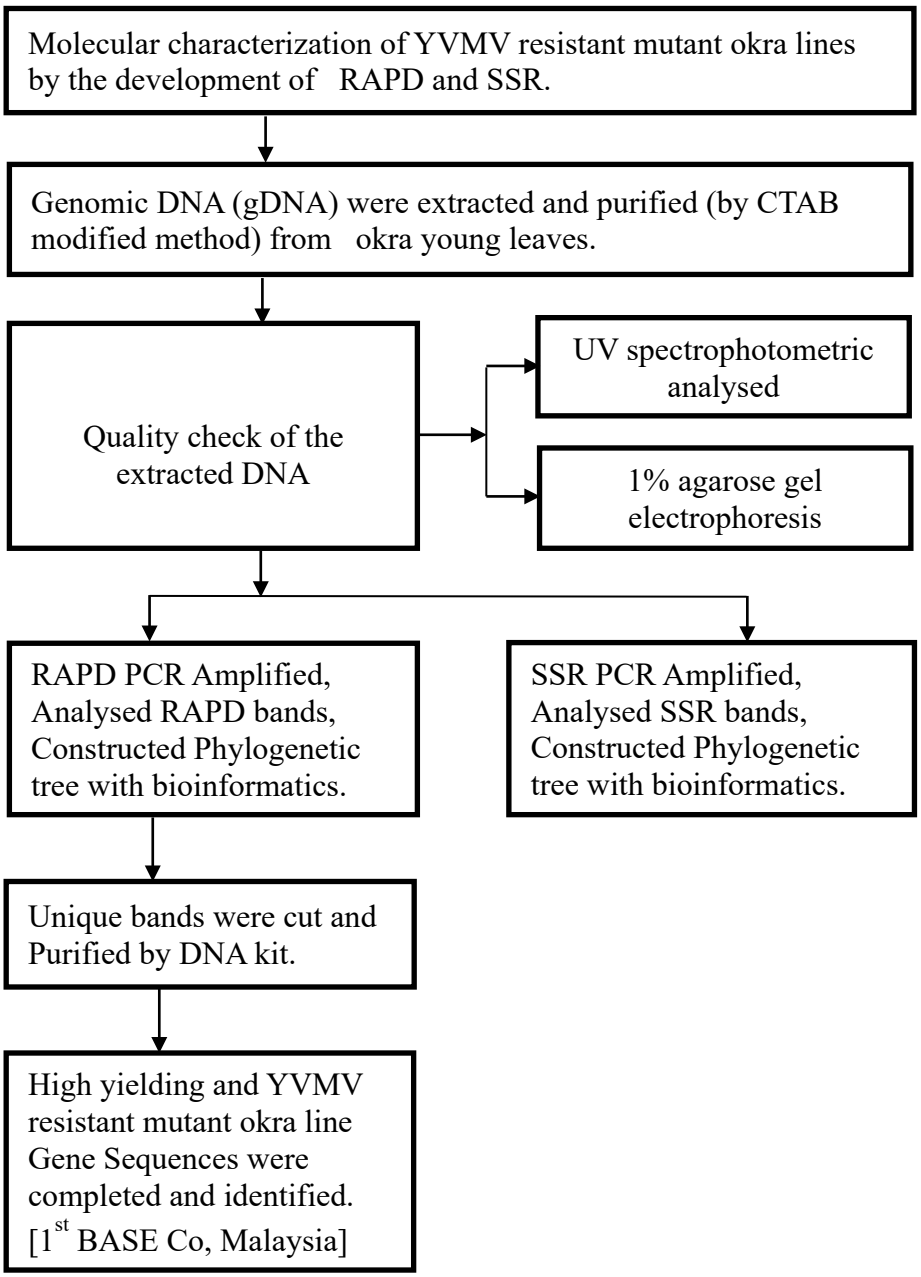

Figure 6.2: Flowchart diagram of materials and methodology.

The details of the laboratory procedures and techniques of methodology followed are given bellow-

6.2.2.1 Isolation of genomic DNA

Leaf samples from the 7 lines with control okra were collected from the Biochemistry Research Garden, University of Rajshahi. Samples were collected in foil paper and cleaned well. The samples were kept in the plastic zipper bag for later use.

6.2.2.2 DNA extraction protocol

Okra leaves are very rich in polysaccharides and phenolics which interfere with the isolation and purification of the genomic DNA. This highly mucilaginous feature of okra plant is the main challenge for achieving good quality DNA. Even after grinding into the liquid nitrogen, the resulting solution was very sticky and made it almost impossible to obtain DNA. This consistency of the okra leaves is because of a large amount of polysaccharides produced during the photosynthesis. The polysaccharide contents also cause problems in PCR by inhibiting certain enzymes. For obtaining PCR quality DNA different methods of DNA extraction were applied and finally, the method described by Doyle & Doyle (1990) gave good quality DNA and also good amplification compared to the other protocols.

6.2.2.2.1 Materials

Equipments used in the experiment are mentioned below

- Eppendorf high-speed centrifuge
- Wise bath
- Micropipette of different ranges
- Vortex mixer
- Deep freezer
- Mortar and pestle
- Gel electrophoresis unit
- UV spectrophotometer
- Centrifuge tubes (2ml and 1.5 ml)
- Icebox

6.2.2.2.2 Reagents required for genomic DNA extraction

Extraction buffer: 20mM Cetyl Trimethyl ammonium Bromide, 100mM Tris Hcl, 20 mM EDTA, 20 mg of Polyvinyl pyrollidine and β-Mercapto ethanol, 2.5 M NaCl.

- Chloroform : Isoamyl alcohol: 24:1
- Phenol : Chloroform : Isoamyl Alcohol: 25: 24: 1
- TE buffer: 10 mM Tris HCl and 1 mM EDTA pH 8.00 were maintained.
- 3 M Sodium Acetate (pH 5.2)
- 2.5 M NaCl
- Ice Cold Isopropanol.
- Wash buffer (0.1M Ammonium Acetate and 76% Ethanol)
- Molecular Grade Ethanol
- RNase A (10 mg/ml)
- TAE buffer (working concentration 1X)
- Bromophenol blue
- Ethidium Bromide (10 mg/ml)

6.2.2.2.3 Components of 2X CTAB buffer

Table 6.2: Components used for preparation of CTAB extraction buffer

Serial	Stock	Final Concentration/amount	Volume (100 ml solution)
01	CTAB	2 g	
02	NaCl	8 g	
03	1 M Tris-HCl	0.1 M	10 ml
04	0.5 M EDTA	0.02 M	4 ml
05	Distilled water		Up to volume

All the solutions were prepared in a sterile condition and pH was maintained properly. All the glassware, reagent tubes, tips were sterilized by autoclave for 20 minutes at 121°C and 15 psi of pressure before use.

6.2.2.2.4 Reagents preparation

a) Preparation of 3 M sodium acetate (pH 5.2; 100 ml)

At first 41 g of sodium acetate was taken in an autoclaved reagent bottle containing 65-70 ml distilled water and dissolved the sodium acetate. pH was adjusted to 5.2 by adding glacial acetic acid. Volume was made up to 100 ml with distilled water by measuring cylinder. The solution was autoclaved and stored at room temperature.

b) Preparation of 0.5 M EDTA (pH 8.0; 250 ml)

46.53 g of EDTA was dissolved in 200 ml of distilled water in an autoclaved reagent bottle. Then the pH was adjusted to 8.0 by adding NaOH pellets. The volume was made up to 250 ml with distilled water by measuring cylinder. The solution was then autoclaved and stored at room temperature.

c) Preparation of 1 M Tris-HCl (pH 8.0; 100 ml)

12.11 g Tris were collected in an autoclaved reagent bottle and dissolved in 80 ml of distilled water by stirring. Then the pH was adjusted to 8.0 by slowly adding HCl. After that, the volume was made up to 100 ml with distilled water. The solution was then autoclaved and stored at room temperature.

d) Preparation of 2X CTAB (100 ml)

First 2 g of CTAB powder was taken in an autoclaved reagent bottle and 8 g of NaCl was added. Then 60-70 ml of distilled water was added and the mixture was dissolved well. And then 0.1M Tris-HCl (10 ml 1M Tris-HCl solution) was added. After that 0.02M EDTA (4 ml of 0.5M EDTA; pH 8.0) was added. Finally the volume was made up to 100 ml by measuring cylinder.

e) Preparation of TE buffer (Tris-EDTA; pH 8.0; 100 ml)

1 ml of 1M Tris-HCl (pH 8.0) and 0.2M of EDTA (pH 8.0) were taken in an autoclaved reagent bottle. Then the pH kept at 8.0 by adding glacial acetic acid. Finally, the volume was made up to 100 ml with distilled water. Finally, the solution was autoclaved and stored at room temperature.

f) Preparation of 0.1 M ammonium acetate (100 ml)

In an autoclaved reagent bottle 0.77 g ammonium acetate was taken in 80 ml of distilled water and mix well. The volume was then made up to 100 ml with distilled water. The solution was then autoclaved at 121°C and stored at room temperature.

g) Preparation of wash buffer (100 ml)

10 ml of 0.1M ammonium acetate was taken in an autoclaved reagent bottle and then 76 ml of 76% ethanol was added. Finally, the volume was made 100 ml by adding distilled water. The solution was stored at room temperature.

h) Preparation of 50 X TAE buffer (250 ml)

60.5 g Tris-base was added in 125 ml distilled water and mixed well in an autoclaved reagent bottle. Then 14.3 g glacial acetic acid was added and stirred well. After that 25 ml of 0.5 M EDTA (pH 8.0) was added and the pH was adjusted to 8.0 with NaOH. Finally, the volume was made 250 ml by autoclaved water. The solution was then autoclaved at 121°C and stored at room temperature.

i) Preparation of 1 X TAE buffer (500 ml)

10 ml of 50X TAE buffer was taken in an autoclaved reagent bottle and then made up to 500 ml with autoclaved water. The solution was stored at room temperature.

6.2.2.2.5 Leaf material use for DNA extraction

Young leaves of 10-15 days were collected and cleaned well. Leaves that are healthy and free from disease were used for the experiment. Samples were collected in an ice box.

6.2.2.2.6 DNA extraction procedure

The extraction procedure was maintained as follows:

1) At first, good and healthy plant leaves are collected
2) 150 g of leaf material was taken individually in previously autoclaved mortar.
3) Then liquid nitrogen was added into the mortar.
4) The degraded samples were then taken into eppendorf individually.
5) 10 mg of Polyvinyl Pyrrolidone (PVP) was taken to each eppendorf tube. Then 1 ml of 2X CTAB was added and vortex well. After that 2 µl of ß mercepto ethanol

was added to each tube. Then the mixtures were incubated for 30 minutes at the water bath at 65ºC.

6) After incubation, 1 ml of the mixture was added to each sample.
7) Then the samples with extraction buffer were incubated for 60 minutes at 65ºC and kept the sample at room temperature for 5 minutes.
8) After cooling at room temperature, 400 µl of Chloroform : Isoamyl Alcohol (24:1) was added and mixed gently.
9) Centrifuged the mixture at 13000 rpm for 5 minutes.
10) Then the upper aqueous layer was transferred to new eppendorf tube (1.5 ml tube).
11) An equal volume of ice-cold isopropanol was added.
12) Incubated at -20ºC for overnight.
13) In the next day, the mixture was centrifuged at 13000 rpm for 2 minutes.
14) Supernatant was discarded and the pellets were washed by adding 1 ml of wash buffer to each eppendorf tube.
15) The mixture was centrifuged again at 13000 rpm for 5 minutes.
16) The supernatant discarded and pellets were air dried and finally, 50 µl of TE buffer was added to resuspened the DNA.

6.2.2.2.7 Purification of okra gDNA

1st Day

1) 0.2 µl RNase (10 mg/ml) was added to each DNA sample.
2) Incubated at 37 ºC for 1 hour to digest the RNA.
3) An equal volume of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added.
4) The mixture was vortexed and centrifuged at 13000 rpm for 15 minutes at 4 ºC.
5) The upper layer was transferred individually in a new 1 ml eppendorf tube.
6) 2/3 volume of ice-cold Isopropanol was added.
7) 0.1 volume of 3 M sodium acetate was added and mixed gently.
8) Then the mixture was incubated overnight at -20°C to precipitate the DNA.

2nd Day

9) Next day the mixture of each eppendorf tube was centrifuged at 13000 rpm for 15 minutes at 4° C.

10) The supernatant was discarded.

11) 500 µl of wash buffer was added to the pellet and centrifuged again at 13000 rpm for 15 minutes at 4 °C.

12) The supernatant was discarded.

13) Pellets were air dried.

14) 50 µl of TE buffer was added to each tube for the resuspension of the DNA.

15) Finally, the purified DNA was stored at -20ºC.

6.2.3 Quantification and quality determination of genomic DNA

6.2.3.1 Spectrophotometric analysis

The amount of DNA of the extracted sample was measured using a UV spectrophotometer at 260 nm. Optical density of the samples was measured and the amount of the DNA was determined by using the standard OD 1=50 µg of DNA.

10 µl of DNA samples was diluted with 990 µl of nuclease-free water in a micro cuvette. OD was taken at 260 nm and 280 nm. The concentration of DNA in the samples were measured using the following formula:

$$\text{DNA } (\mu g/\mu l) = \frac{OD_{260} \times 50 \times \text{dilution factor}}{1000}$$

$$\text{Dilution factor} = \frac{\text{Volume made}}{\text{The volume of the aliquot}}$$

$$\text{Therefore, Dilution factor} = \frac{1000\ \mu l}{10\ \mu l} = 100$$

The purity of the DNA was determined by measuring the ratio of OD_{260}/OD_{280}. 260/280 ratio value of highly purified DNA ranges from 1.7~2.0.

6.2.3.2 Agarose gel electrophoresis

Quality of the extracted DNA was checked by 1% agarose gel electrophoresis with 1X TAE buffer. About 3µl of extracted DNA from each line okra was mixed with 1µl of loading dye and applied onto the gel. Electrophoresis was carried out by using a power supplier at 70 V for 30 minutes. The gel was then removed from the gel chamber and viewed under the UV transilluminator to visualize the DNA bands. The physical integrity of the bands was measured from the agarose gel electrophoresis.

6.2.3.2.1 Requirements

- Electrophoresis unit, gel combs, gel casting tray, power supplier (Life Technologies)
- Microwave oven
- Gel documentation unit (Cleaver science)
- Agarose
- 50X TAE buffer (Stock solution)
- 1X TAE buffer (Working solution)
- Ethidium bromide (10mg/ml)

6.2.3.2.2 Procedure

At first, agarose was weighed according to the size of the gel to be prepared. 1% agarose gel was prepared for electrophoresis. Then agarose was dissolved in 1X TAE by heating the solution in Microwave oven for 1 minute. The solution was then cooled to 60°C and Ethidium bromide (10 mg/ml) was added. The solution was mixed properly and was poured into the gel casting tray after placing the comb into the tray. The gel was poured into the tray very carefully for preventing the formation of bubbles. After the gel solidified, the comb was removed carefully. The solidified gel was then transferred to the electrophoresis unit and a sufficient amount of 1X TAE buffer was poured that coverd the wells completely.

The purified DNA with loading dye (Bromophenol Blue) was then applied to the wells of the gel with the help of an adjustable micropipette. Power supplier was

switched on and 70 V was applied for 30 minutes to run the DNA. After the completion of electrophoresis, the gel was viewed on gel documentation system (Cleaver Science).

The flowchart of preparation of 30 ml 1% agarose gel:

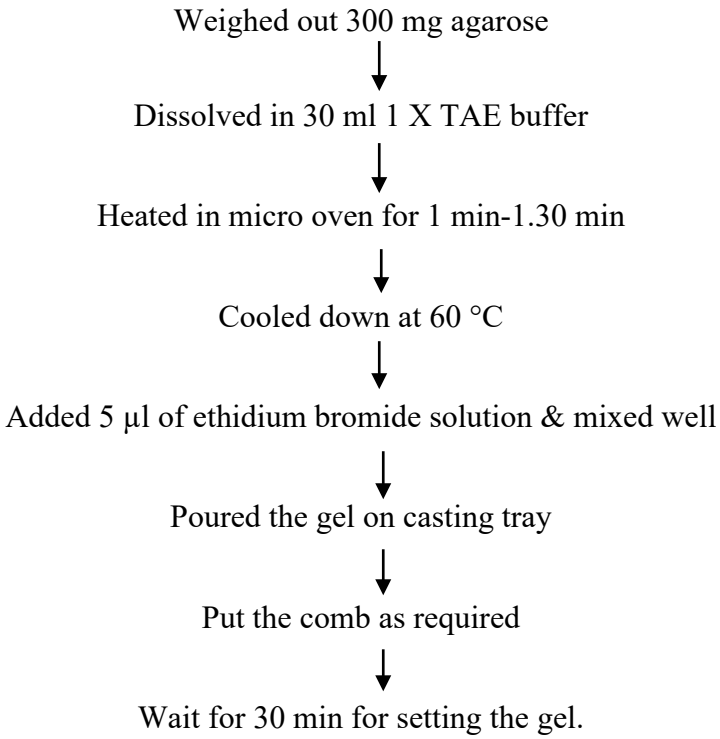

Figure 6.3: Flowchart diagram of materials and methodology for the preparation of 30 ml 1% agarose gel.

6.2.4 RAPD - PCR amplification

Following parameters were optimized during PCR reaction.

- Template DNA concentration
- Primer concentration
- Primer annealing temperature
- Taq DNA polymerase concentration
- Selection of suitable primer for detection of polymorphic loci.
- Optimization of the PCR condition

As PCR is a very sensitive reaction, sterile condition was properly maintained during the preparation of the reaction mixture. Autoclaved double distilled water, Pipette tips, PCR tubes were used for the reaction to avoid any type of contamination.

6.2.4.1 Instruments required for PCR

- Thermal cycler
- Microcentrifuge tubes
- Auto pipettes of ranges from 0.5-10 µl,10-100 µl and 1-1000 µl
- Micropipette tips
- Deep freezer (-20°C)
- Ice Box
- Electrophoresis unit with power supplier.

6.2.4.2 PCR contents

Table 6.3: PCR composition for RAPD amplification

Si No	Contents	Concentration of the contents	Volume in 10 µl mixture
1	DNA polymerase master mix	2X	5µl
2	DNA template	50ng/µl	1µl
3	RAPD Primer	10µM	0.5µl
4	Nuclease-free water		3.5µl
5	Total volume		10µl

6.2.4.3 PCR procedure

1. At first, all the reagents and DNA samples were kept in ice.
2. The final mixture was prepared and pipetting was done accurately.
3. All the sample tubes were mixed uniformly by gently tapping the tube and by repeated pipetting.
4. Thermocycler was switched on at least 15 minutes earlier.
5. Now the sample tubes were firmly placed on the lids of the thermocycler.
6. Amplification condition was programmed at the final stage.

6.2.4.4 PCR condition

PCR condition was optimized several times using different protocols. Finally, the protocol described by Williams *et al*. (1990) and Welsh & Mc Clelland (1990) gave good amplification and scorable RAPD bands.

The optimum condition of amplification for each of the RAPD primer consists of the following three steps which were repeated 46 times.

Table 6.4: Amplification condition for RAPD-PCR

Amplification step	Amplification temperature (°C)	Time (minute)
Initial denaturation	94	5.00
Denaturation	94	1.00
Primer annealing	36	00.30
Extension	72	3.00
Final extension	72	5.00
Cooling	4	10.00

46 cycles

6.2.4.5 Resolution of amplified product on agarose gel electrophoresis

PCR Amplified product was visualized on 1% agarose gel. The gel was prepared as the procedure described before. 3 µl of the amplified product was then applied into the gel wells. After the completion of electrophoresis, the gel was viewed under the gel documentation system. RAPD band was observed and detected for further analysis.

6.2.4.6 Analysis of RAPD profile

PCR Amplification pattern for all the primer used was compared with each other and the bands of the DNA fragments were scored as presence (1) or absence (0) generating the 0,1 matrices. The presence or absence of data obtained from each primer were analysed with the online computer software Dendro UPGMA. The clustering and dendrogram were constructed using the Jaccard Coefficient method (Chauhan *et al*., 2017; Jaccard, 1908).

Percent of Polymorphism and Discrimination power was analysed using the following formula:

$$\text{Percent (\%) polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

$$\text{Discrimination power} = \frac{\text{Number of unique RAPD bands}}{\text{Total number of RAPD bands}} \times 100$$

6.2.5 SSR-PCR amplification

For amplification of DNA with SSR primers, the following parameters were optimized.

- The Purity of the genomic DNA
- The concentration of genomic DNA
- The concentration of SSR primer
- Annealing temperature
- Taq DNA polymerase concentration
- Selection of suitable primer for detection of polymorphic loci.
- Optimization of the PCR condition.

6.2.5.1 PCR contents

Table 6.5: PCR composition for SSR amplification

Contents	Concentration of the contents	Volume in 10 µl mixture
DNA polymerase master mix	2X	5µl
DNA template	50 ng/µl	1µl
Forward primer	10 µM	0.5µl
Reverse primer	10 µM	0.5µl
Nuclease-free water		3µl
Total volume		10 µl

6.2.5.2 PCR condition

PCR condition was optimized several times with different temperature profiles. Finally, the PCR condition was described by (Sawadogo *et al*., 2009).

The temperature profile for each of the SSR primer is as follows:

Table 6.6: Cycling temperature for SSR-PCR amplification

Amplification step	Amplification temperature (°C)	Time (minute)
Initial denaturation	95	5.00
Denaturation	94 (35 cycles)	00.30
Primer annealing	55 (35 cycles)	00.45
Extension	72 (35 cycles)	1.00
Final extension	72	7.00
Cooling	4	10.00

6.2.5.3 Resolution of amplified product on agarose gel

PCR Amplified product was visualized on 1% agarose gel. The gel was prepared as the procedure described before. 3 µl of the amplified product was then applied into the gel wells. After the completion of electrophoresis, the gel was viewed under the gel documentation system. SSR band was observed and detected for further analysis.

6.2.5.4 Analysis of SSR profile

PCR Amplification pattern for all the primer used was compared with each other and the bands of the DNA fragments were scored as presence (1) or absence (0) generating the 0,1 matrices. The presence or absence of data obtain from each primer were analyzed with the online computer software Dendro UPGMA. The clustering and dendrogram was made using the Jaccard coefficient method (Jaccard, 1908; Kaur *et al*., 2018).

Percent of Polymorphism and Discrimination power will be analysed using the following formula

$$\text{Percent (\%) polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

$$\text{Discrimination power} = \frac{\text{Number of unique SSR bands}}{\text{Total number of SSR bands}} \times 100$$

Table 6.7: Random decamer used for the genetic diversity study

Serial no.	Primer code	Primer sequence (5'~3')	Molecular weight
01	OPA 01	CAGGCCCTTC	2964
02	OPA 02	TGCCGAGCTG	3044
03	OPA 03	AGTCAGCCAC	2997
04	OPA 04	AATCGGGCTG	3068
05	OPA 05	AGGGGTCTTG	3099
06	OPA 06	GGTCCCTGAC	3004
07	OPA 07	GAAACGGGTG	3117
08	OPA 08	GTGACGTAGG	3108
09	OPA 09	GGGTAACGCG	3053
10	OPA 10	GTGATCGCAG	3068
11	OPA 11	CAATCGCCGT	2988
12	OPA 12	TCGGCGATAG	3068
13	OPA 13	CAGCACCCAC	2942
14	OPA 14	TCTGTGCTGG	3050
15	OPA 15	TTCCGAACCC	2948
16	OPA 16	AGCCAGCGAA	3046
17	OPA 17	GACCGCTTGT	3019
18	OPA 18	AGGTGACCGT	3068
19	OPA 19	CAAACGTCGC	3037
20	OPA 20	GTTGCGATCC	3019

Table 6.8: SSR marker used for genetic diversity studies

Sl. No.	Primer code	Primer sequence	No of bases
1	SSR 9	F-ACCTTGAACACCAGGTACAG	20
		R-TTGCTCTTATGAAGCAGTGA	20
2	SSR 66	F-CACCAGAATTTCCCTTTTG	19
		R-ACTGTTGTTTGGCTTATGCT	20
3	SSR 64	F-AAGGAGGAGAAAGAGAAGGA	20
		R-ATTACTTGAGCAGCAGCAG	19
4	SSR 89	F-TTTGAGTTCTTTCGTCCACT	19
		R-GTATTTGGACATGGCGTTAT	20
5	SSR 52	F-AACACATCCTCATCCTCATC	20
		R-ACCGGAAGCTATTTACATGA	20
6	SSR 63	F-GTGTTTGAAAGGGACTGTGT	20
		R-CTTCATCAAAACCATGCAG	19
7	SSR 78	F-CTCCGACAATTCAAGAAAAG	20
		R-CACCCAATCAAGCTATGTTA	20
8	SSR 56	F-GGCAACTTCGTAATTTCCTA	20
		R-TGAGTAAAAGTGGGGTCTGT	20
9	SSR 54	F-CGAAAAGGAAACTCAACAAC	20
		R-TGAACCTTATTTTCCTCGTG	19

6.2.6 Unique bands purification and characterization

The Unique Band was obtained by using RAPD primer (OPA 9 and OPA 11), of which detailed materials and methods were described before.

Unique RAPD bands were cut and purified by DNA kit details procedure as shown bellow:

Previously got unique band PCR product 4 µl was used to make upto 40 µl as new PCR product. Again 3 µl (from 40 µl) was used to 1% Agarose gel to confirm the unique band. After reconfirmation of the unique band, gel was prepared to run 30-40 µl new PCR products into the gel. By this time, preparation was completed to cut the gel.

After completion of the gel electrophoresis function, the gel was transferred to the gel documentation system and the unique band was visualized under uv light and the gel was cut with unique bands and then was carefully kept into the eppendorf tube. These materials were carried out for purification.

The PCR product was purified through recovery from the gel. It was carried out by the GF-1 Gel DNA Recovery Kit (Vivantis Co Ltd, Malaysia). Details of the procedure of purification are as follows:

1. The PCR products were run in gel and desired bands were cut from the gel.
2. Then these cutting parts of gel were taken into Eppendorf tube and weighted.
3. GB buffer (Vivantis Co. Ltd.) was added (1 volume GB: 1 volume gel).
4. Incubated at 50 °C for 5-7 min until the gel melted completely.
5. Transferred into the loading column.
6. Centrifuged at 10,000 rpm for 1 min.
7. The supernatant was discarded.
8. 750 µl wash buffer (Vivantis Co. Ltd) was added.
9. Centrifuged at 10,000 rpm for 1 min.
10. Centrifuged again at 10,000 rpm for 1 min.
11. Left for 5 mins.
12. Filter (column) was placed into a new autoclaved Eppendorf tube.
13. 30-50 µl Elution buffer (Vivantis Co. Ltd.) was added and left for 2 min.
14. Centrifuged again at 10,000 rpm for 1 min.
15. The purified DNA was stored at -20 °C.

Finally, the purified DNA samples were checked through 1% agarose gel electrophoresis and the procedure sample prepared at least 20 µl for DNA sequencing was followed.

6.2.7 DNA sequencing and Bioinformatic analysis

Purified sample was tagged and packed properly with respective primer tube. Packet was handedover to the responsible authority for abroad for DNA sequencing.

After getting of the gene sequence result, software (by BLAST search) was used for gene identification.

6.3 Results and Discussion

6.3.1 Genetic diversity analysis among the mutant 7 lines of okra using RAPD marker and SSR marker

6.3.1.1 Isolation and quantification of genomic DNA

Genomic DNA extraction of Mutant 7 lines with Control of okra was carried out by following the protocol described by Doyle & Doyle., 1990. A minor modification of this protocol gives good quality DNA for PCR amplification. Doyle & Doyles method is good for extraction of genomic DNA of other plants. But while extracting genomic DNA from okra leaves, we have faced several problems. Okra is a highly mucilaginous plants and high phenolic substance interfere with the DNA extraction. The physical integrity of DNA on 1% agarose gel was good but the DNA has contaminants and when this DNA was subjected to PCR amplification, it did not show any amplification. The genomic DNA bands were also very faint. After resuspenson in TE buffer it appears yellowish colour. Doyle's method used 1.4 M NaCl for the preparation of CTAB extraction buffer. In the following experiment, we have used 2.5 M NaCl in the extraction buffer. Again Doyles method used 30 mins incubation at 65°C and finally they have used 70% ethanol as wash buffer but in this experiment 60 minutes incubation at 65°C were optimum for okra genomic DNA extraction. 70% absolute ethanol was used as wash buffer. PCR protocols were optimized several times with different methods for the removal of these PCR inhibitors. Finally, the CTAB buffer method described by Doyle & Doyle (1990) gave high-quality DNA and clearly amplifiable genomic DNA. This minor modification helps isolate mucilage free DNA from okra leaf and this genomic DNA was amplified by PCR. The quality of the extracted DNA was measured by 1% agarose gel electrophoresis and the quantity of the DNA was measured by a UV-vis spectrophotometer with an emission spectrum at 260 nm.

DNA samples were diluted 100 times with double distilled water and applied to the spectrophotometer at 260 nm. DNA was also quantified at 260 nm/280 nm to measure the purity of the extracted DNA. $OD_{260/280}$ ratio determines the purity of the DNA. Ratio value between 1.7~2.00 indicates highly purified DNA free from PCR inhibitors. The quantity of the DNA was varied from 895 ng/µl to 1732 ng/µl. The

physical integrity of the DNA was also found pretty well by 1% agarose gel electrophoresis to carry out the PCR amplification. The sharp band was found at >10000 bp through 1% agarose gel electrophoresis (Figure 6.4).

Different protocols have been established for the extraction of genomic DNA from plants which have high mucilage content and other phenolic substances such as Dellaporta *et al*., 1983; Bernatzky & Tanksley, 1986; Geuna *et al*., 2003; Doyle & Doyle., 1990; Kim *et al*., 1997; Lodhi *et al*., 1994: Li *et al*., 2002; but these did not give satisfactory results with okra.

Singh *et al* (2017), discussed the modification of genomic DNA extraction protocol for mucilage rich plant like okra. They also reported that minor modification of the extraction protocol gives good and sharp bands.

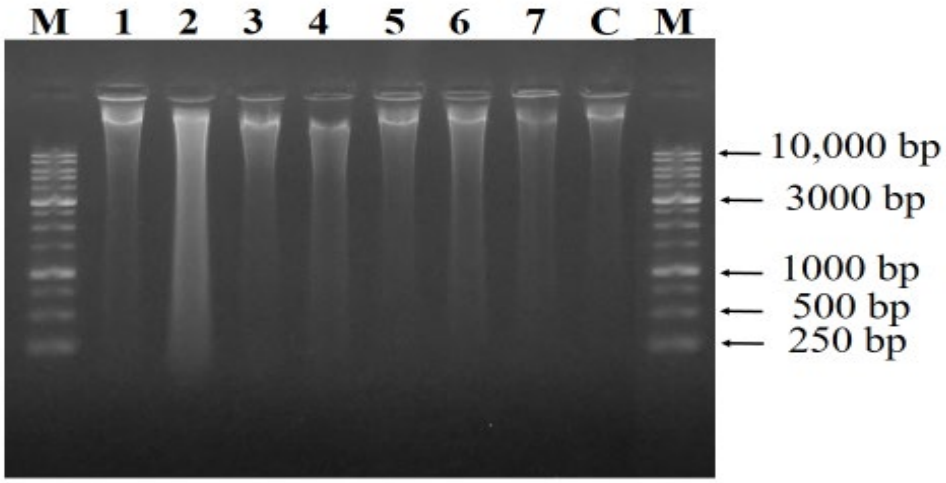

Figure 6.4: Genomic DNA of YVMV resistant 7 lines mutant with control of okra in 1% agarose gel.

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

6.3.1.2 Genomic DNA quantity and quality of okra genotypes

The concentration of the entire genomic DNA was measured spectrophotometrically. Procedures for the quantification of DNA were described in the materials and methodology. Amount of DNA present in each variety is described in the Table 6.9

Table 6.9: Concentration of genomic DNA among different individuals

SL. No.	Name of Lines	OD_{260}/OD_{280} Ratio	Concentration (ng/µl)
1	Mutant 5 Line 1 = M5L1	1.92	1250
2	Mutant 5 Line 2 = M5L2	1.88	1732
3	Mutant 5 Line 3 = M5L3	1.86	1550
4	Mutant 5 Line 4 = M5L4	2.45	1000
5	Mutant 5 Line 5 = M5L5	1.82	895
6	Mutant 5 Line 6 = M5L6	1.95	1000
7	Mutant 5 Line 7 = M5L7	2.25	1450
8	Control -Shamol Bangla-C.	1.98	1150

6.3.1.3 PCR amplification

PCR amplification was carried out using random decamer primers (RAPD) and SSR with Mutant 7 lines with Control (8 individuals) of *Abelmoschus esculentus.* The reaction condition and reaction components were optimized to yield highly scorable and clear RAPD & SSR amplified fragments. After the completion of PCR reaction, the amplified products were separated on 1% agarose gel electrophoresis which showed good resolution for scoring the bands.

6.3.1.4 Genetic relationship among the mutant 7 lines with control of okra using RAPD marker

6.3.1.4.1 PCR amplification

PCR amplification was carried out using 20 random primers with 8 individuals of *Abelmoschus esculentus*. Out of 20 random decamer primers, 10 gave scorable amplification with good polymorphism and 2 gave monomorphic (unique band) band (Table 6.10). Reproducible RAPD fragments can be obtained if the cycling

parameters and reaction composition are properly mixed without any alteration. So for the optimization of the amplification of okra genomic DNA, several cycling temperatures and reaction components were tested. Therefore 1 µl (50 ng) of template DNA, 0.5 µl of 10 µM primer, 5 µl of taq master mix and 3.5 µl of nuclease-free water for 10 µl reaction volume were used for the amplification. A denaturation at 94°C, Primer annealing at 36°C, extension temperature at 72°C was found optimum for the RAPD-PCR amplification.

PCR condition, PCR components and cycling parameters were optimized depending on the experimental samples and lab facilities. Different studies used different cycling temperatures.

Ikram *et al*., 2013 analysed genetic diversity of twenty nine okra accessions by RAPD primer using annealing temperature 36°C. As RAPD primers are short oligonucleotide of 10 bp and for this reason low annealing temperatures from 36°C to 40°C are used for this type of experiment. Prakash *et al*., 2011 studied genetic diversity of okra by RAPD primer using annealing temperature at 40°C.

6.3.1.4.2 Analysis of RAPD profiles

PCR amplification was carried out using 20 random primers with 8 individuals of *Abelmoschus esculentus*. Out of 20 random decamer primers, 10 gave scorable amplification with good polymorphism and 2 gave monomorphic (unique band) band. RAPD profiles were calculated using these 10 primers (Table 6.10) and 2 primers were used for unique band analysis in gene sequence part. The primers that gave good amplification with almost all the genotypes were only selected for this experiment to construct dendrogram.

A few genotypes did not give good amplification with a few primers. It was ensured that the DNA samples were intact and their genomic DNA bands were also pretty good. PCR conditions were uniform for all the 8 genotypes studied. It has also seen that some of the genotypes which did not amplify with one primer gave good amplification with the other primer. This kind of information obtained from the DNA fingerprint could be used for identification of a particular genotype. The protocol for RAPD analysis was optimized several times with respect to template DNA

concentration, primer concentration, a master mix containing taq DNA polymerase. It was found that a primer concentration of 10 μM, 25-30 ng of template DNA, and a master mix of 2X concentration are optimum for RAPD PCR amplification. The amplified products were then separated and visualized by electrophoresis in 1% agarose gel with ethidium bromide staining.

The number of amplified bands per primer was variable and fragments number was varied between 8 (OPA 13) to 1 (OPA 07 and OPA 20). The size range of the amplified product is also different for each primer. Different fragments were found to be amplified between 150 bp to 1500 bp.

Table 6.10: Random decamer primer (RAPD) used in the experiment for their efficiency to amplify genomic DNA for polymorphism study

Serial No	Primer code	Molecular weight	Total number of bands amplified	Polymorphic bands		PIC
				Number	Frequencies	
1	OPA 01	2964	7	6	85.71	0.464
2	OPA 03	2997	3	2	66.66	0.353
3	OPA 07	3117	2	1	50.00	0.50
4	OPA 08	3108	5	3	60.00	0.430
5	OPA 13	2942	8	7	87.50	0.335
6	OPA 15	2948	4	3	75.00	0.382
7	OPA 16	3046	4	3	75.00	0.398
8	OPA 18	3068	3	3	100.00	0.468
9	OPA 19	3037	3	3	100.00	0.353
10	OPA 20	3019	1	1	100.00	0.468
11	**OPA 09**	**3052**	**3**	**Monomorphic (Unique band)**		-
12	**OPA 11**	**2988**	**3**	**Monomorphic (Unique band)**		-
Total (Calculated value from Sl no 1 to 10)			40	32	80.00	0.415

6.3.1.4.3 Banding pattern of different RAPD primers

RAPD fragments produced by different primers with different mutant lines of okra genotypes are described in the Table 6.10. The number of bands differs with each primer. A total of 40 RAPD bands were formed by the 10 RAPD primers within the mutant 7 lines genotypes of okra. This data was used for further analysis of genetic diversity.

The average number of band was 4.0 per primer. Highest band number was obtained by the primer OPA 13 and number of the band was 8. Lowest number of the band was obtained by both OPA 07 and OPA 20 (1 band). Out of the 40 scorable RAPD bands, 32 bands were found polymorphic. An average number of polymorphic bands for each primer was found 3.2. Among all the primers least polymorphism was obtained by one primer OPA 07 (50.00%) and 100% polymorphism was obtained by three primer OPA 18, OPA 19, OPA 20. About 80% polymorphism was found among the 7 lines. Average PIC value was calculated to be 0.415.

The number of amplified bands per primer was variable and fragments number was varied between 8 (OPA 13) to 1 (OPA 07 and OPA 20). The size range of the amplified product is also different for each primer. Different fragments were found to be amplified between 150 bp to 1500 bp. The major advantage of RAPD marker is that this randomly designed primer can bind to the homologous sequence anywhere in the whole genome providing greater genetic diversity (Williams *et al*., 1990). The RAPD bands obtained by different primers were different from each other because of the change of the DNA sequence of the random decamer primer or reaction condition. The highest band number was obtained by the primer OPA 13 and number of the band was 8 of which 7 bands were found polymorphic (87.50%).

The polymorphism was an indication of prevalence of moderate diversity among the 39 okra genotypes (Punitha & Raveendran, 2004). The cluster analysis (phylogenetic tree) showed a wide range of similarity ranging from 15 to 100% while 86 to 100% genetic similarity was observed using Sequence-related amplified polymorphism (SRAP) markers in okra among 8 genotypes (Gulsen *et al*., 2007). The variation of the amplified banding pattern as revealed by the way of RAPD results in an indication of polymorphism and indicates that there was variation amongst the accessions tested

in this study. The amplification product of the okra accession DNA exhibits specific genetic variations among the accessions. This agrees with the record of Ogunbayo *et al*. (2005) and Nwangburuka *et al*. (2011).

RAPD profiles of different primers are different from each other for their difference of DNA sequences at different loci. The detailed banding patterns of different primers are described below:

Primer OPA 01

Primer OPA 01 gave 7 bands in total and 6 bands of them were polymorphic. Line 1,3,5 and C genotypes gave good amplification with this primer. OPA 01 gave clear RAPD fragments. Fragment size varies from 250 bp to 1300 bp in length. Percent of polymorphism for this primer was calculated as 85.71% (Figure 6.5).

Primer OPA 03

OPA 03 primer gave clear and scorable RAPD fragments in all lines except line 2. Total 3 bands were seen in this primer and 2 of them were found polymorphic. 66.66% polymorphism obtained for this primer and product size was ranged between 200 bp to 900 bp (Figure 6.6).

Primer OPA 07

Primer OPA 07 showed 50.00% polymorphism. Total 2 bands were seen in this primer and 1 of them were found polymorphic. Line 1, 5, 6 and 7genotypes gave good amplification with this primer. The size range of the amplified product varies between 200 bp to 500 bp (Figure 6.7).

Primer OPA 08

Primer OPA 08 showed 60.00% polymorphism. Total 5 bands were seen in this primer and 3 of them were found polymorphic. All the line genotypes gave good amplification except line 2 with this primer. The size range of the amplified product varies between 150 bp to 1100 bp (Figure 6.8).

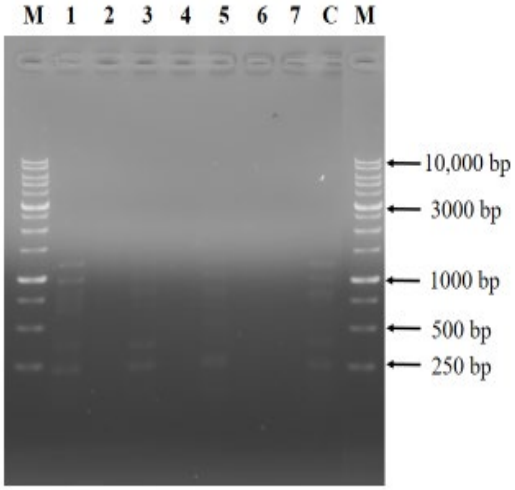

Figure 6.5: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 01.

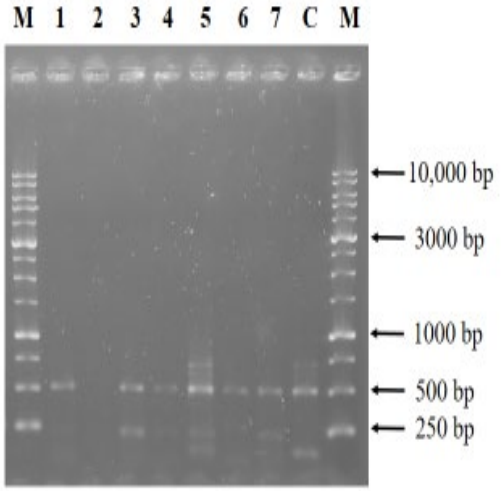

Figure 6.6: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 03.

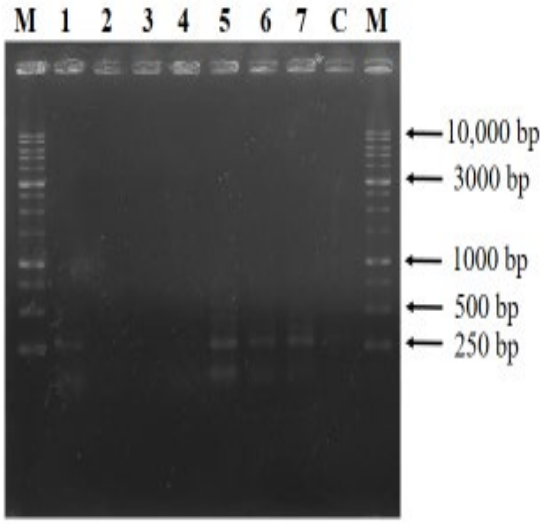

Figure 6.7: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 07.

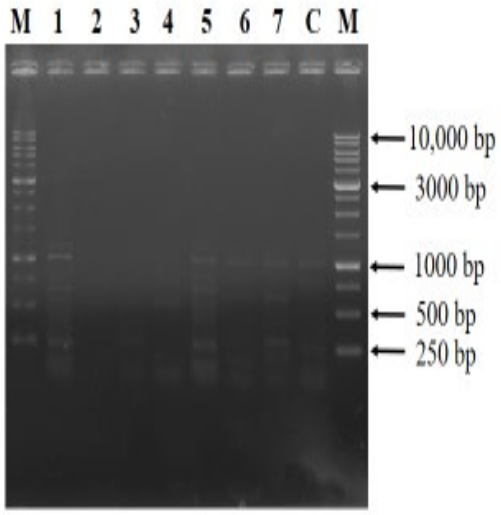

Figure 6.8: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 08.

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

Primer OPA 13

Highest number of bands was obtained by this primer and 8 bands were found. Also found the top most clear band. Primer OPA 13 showed 87.50% polymorphism. Total 8 bands were seen in this primer and 7 of them were found polymorphic. All the line genotypes gave good amplification except line 2 with this primer. The size range of the amplified product varies between 200 bp to 1500 bp (Figure 6.9).

Primer OPA 15

Primer OPA 15 showed 75.00% polymorphism. Total 4 bands were seen in this primer and 3 of them were found polymorphic. Line 1, 5, 6,7 and C genotypes gave good amplification with this primer. The size range of the amplified product varies between 300 bp to 1200 bp (Figure 6.10).

Primer OPA 16

Primer OPA 16 showed 75.00% polymorphism. Total 4 bands were seen in this primer and 3 of them were found polymorphic. All the line genotypes gave good amplification except line 2 and 4 with this primer. Most of the bands were found clear. The size range of the amplified product varies between 300 bp to 1500 bp (Figure 6.11).

Primer OPA 18

A total of 3 bands were amplified by OPA 18 and all of them were found polymorphic. Line 1, 5 and 7 genotypes gave good amplification with this primer. 100% polymorphism obtained by this primer. Fragment length was varied between 250 bp to 750 bp (Figure 6.12).

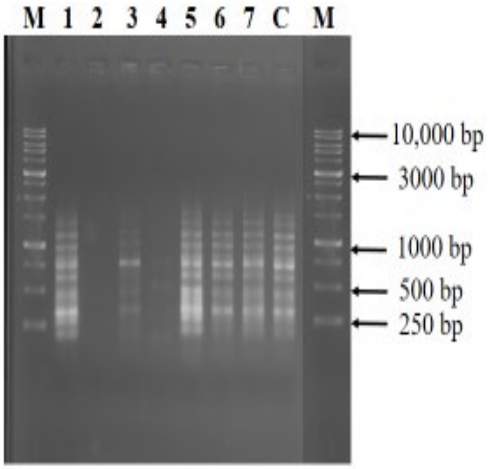

Figure 6.9: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 13.

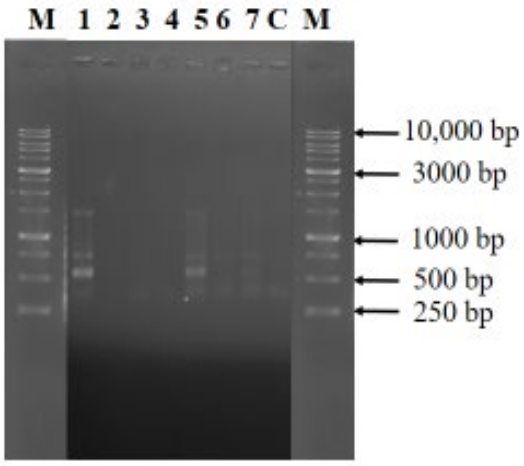

Figure 6.10: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 15.

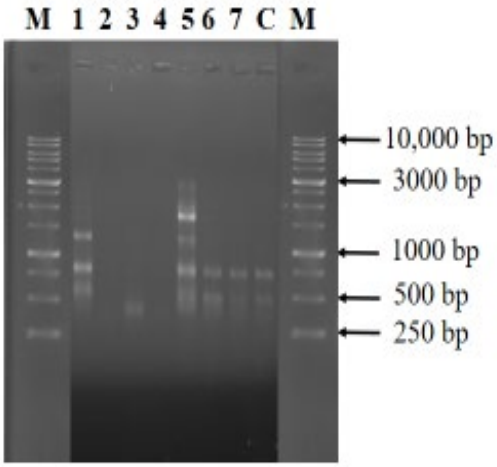

Figure 6.11: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 16.

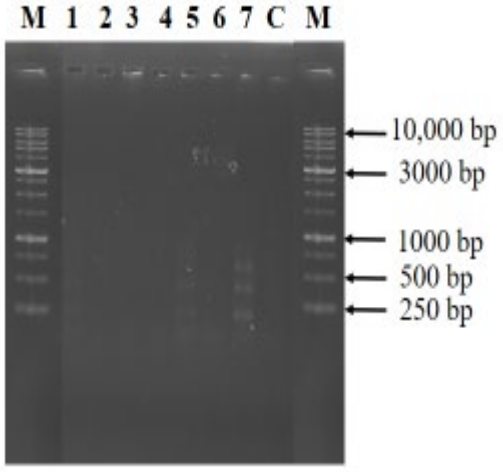

Figure 6.12: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 18.

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

Primer OPA 19

A total of 3 bands were amplified by OPA 19 and all of them were found polymorphic. All lines genotypes gave good amplification except line 2 with this primer. 100% polymorphism obtained by this primer. Fragment length was varied between 250 bp to 750 bp (Figure 6.13).

Primer OPA 20

Lowest number of bands was obtained by this primer and 1 band was found. Primer OPA 20 showed 1 band and 1 of them were found as polymorphic. Line 2, 3, 5, 6 and 7 genotypes gave good amplification with this primer. 100% polymorphism obtained by this primer. Product size range varied between 250 bp to 300 bp (Figure 6.14).

Primer OPA 09

1 band was obtained by this primer. Line 5 and 7 genotype showed the band. This was the monomorphic band which is also known as unique band. The unique bands were found very much sharp. Fragment length was varied between 500 bp to 700 bp (Figure 6.15).

Primer OPA 11

3 bands were obtained by this primer only in line 5 genotype and this was the monomorphic band which is also known as unique band. The unique bands were found very much sharp. Line 7 also gave a band. Fragment length was varied between 400 bp to 1300 bp (Figure 6.16). This line (M5L5) 3 bands were cut for purification and as well as identification.

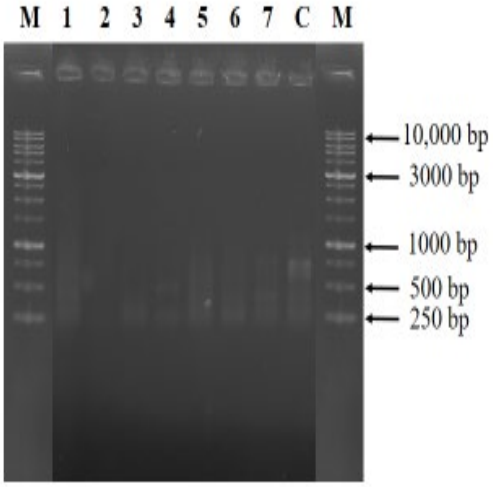

Figure 6.13: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 19.

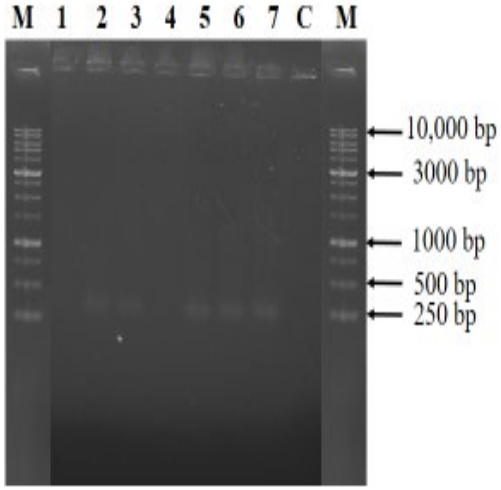

Figure 6.14: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 20.

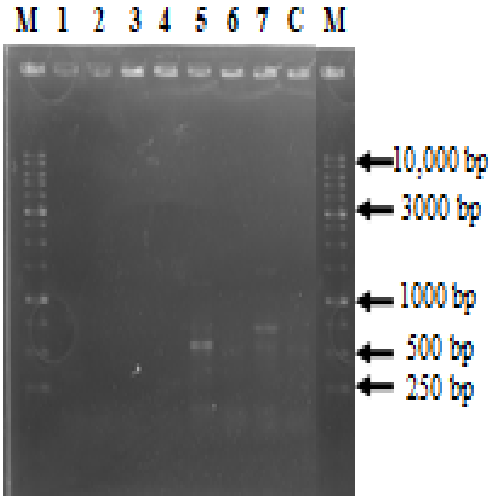

Figure 6.15: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 09.

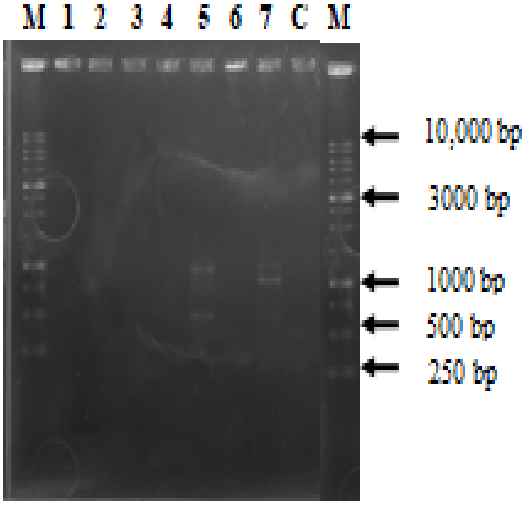

Figure 6.16: RAPD profiles of Mutant 7 lines with control of okra genotypes using primer OPA 11.

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

6.3.1.4.4 Phylogenetic tree analysis using RAPD marker

For genetic diversity analysis first a binary data matrix was prepared. RAPD bands for all the primers were observed manually for detecting the presence or absence of bands for particular locus. While manually inspecting the RAPD images, the presence of bands was scored as 1 and absence of bands for a particular locus was scored as 0. Thus a binary matrix was constructed for statistical analysis of the bands.

The data generated by RAPD marker was used for the genetic diversity analysis. All the 40 RAPD fragments were used for the present analysis of genetic diversity of mutant 7 lines with control okra genotypes. Jaccard coefficient method was applied for the construction of the phylogenetic tree. Distance matrix value indicates a narrow genetic diversity among the okra genotypes. The primary cause for the narrow genetic relationship between the different okra lines is their cross-pollination. Prakash *et al.* (2011) studied the genetic diversity of okra by RAPD marker and reported narrow genetic distances between the individuals.

Statistical analysis of the banding pattern of all the RAPD markers led to the development of a dendrogram on the basis of the genetic distances among different individuals of mutant okra. The dendrogram was prepared using an online software package called Dendro UPGMA and clustering was done using the Jaccard index (Bruel *et al*., 2006).

Phylogenetic rooted tree showed C is very close to M5L3and far from M5L2. On the other hand, M5L5 & M5L1, M5L6 & M5L7, M5L4 & M5L2 were genetically in close relationship to each other (Figure 6.19).

Although the banding pattern of different okra genotypes was different, further powerful techniques are required for the appropriate identification and discrimination of different varieties. Further confirmatory studies should be done using a large number of markers before the firm inference of the varieties.

	M5L1	M5L2	M5L3	M5L4	M5L5	M5L6	M5L7	C
M5L1	1	0	0.486	0.229	0.85	0.528	0.658	0.595
M5L2		1	0.05	0	0.026	0.05	0.036	0
M5L3			1	0.217	0.475	0.429	0.412	0.63
M5L4				1	0.205	0.4	0.286	0.28
M5L5					1	0.513	0.718	0.615
M5L6						1	0.714	0.571
M5L7							1	0.486
C								1

Figure 6.17: RAPD similarity matrix of mutant 7 lines with control of okra.

	M5L1	M5L2	M5L3	M5L4	M5L5	M5L6	M5L7	C
M5L1	0	1	0.514	0.771	0.15	0.472	0.342	0.405
M5L2		0	0.95	1	0.974	0.95	0.964	1
M5L3			0	0.783	0.525	0.571	0.588	0.37
M5L4				0	0.795	0.6	0.714	0.72
M5L5					0	0.487	0.282	0.385
M5L6						0	0.286	0.429
M5L7							0	0.514
C								0

Figure 6.18: RAPD distance matrix of mutant 7 lines with control of okra.

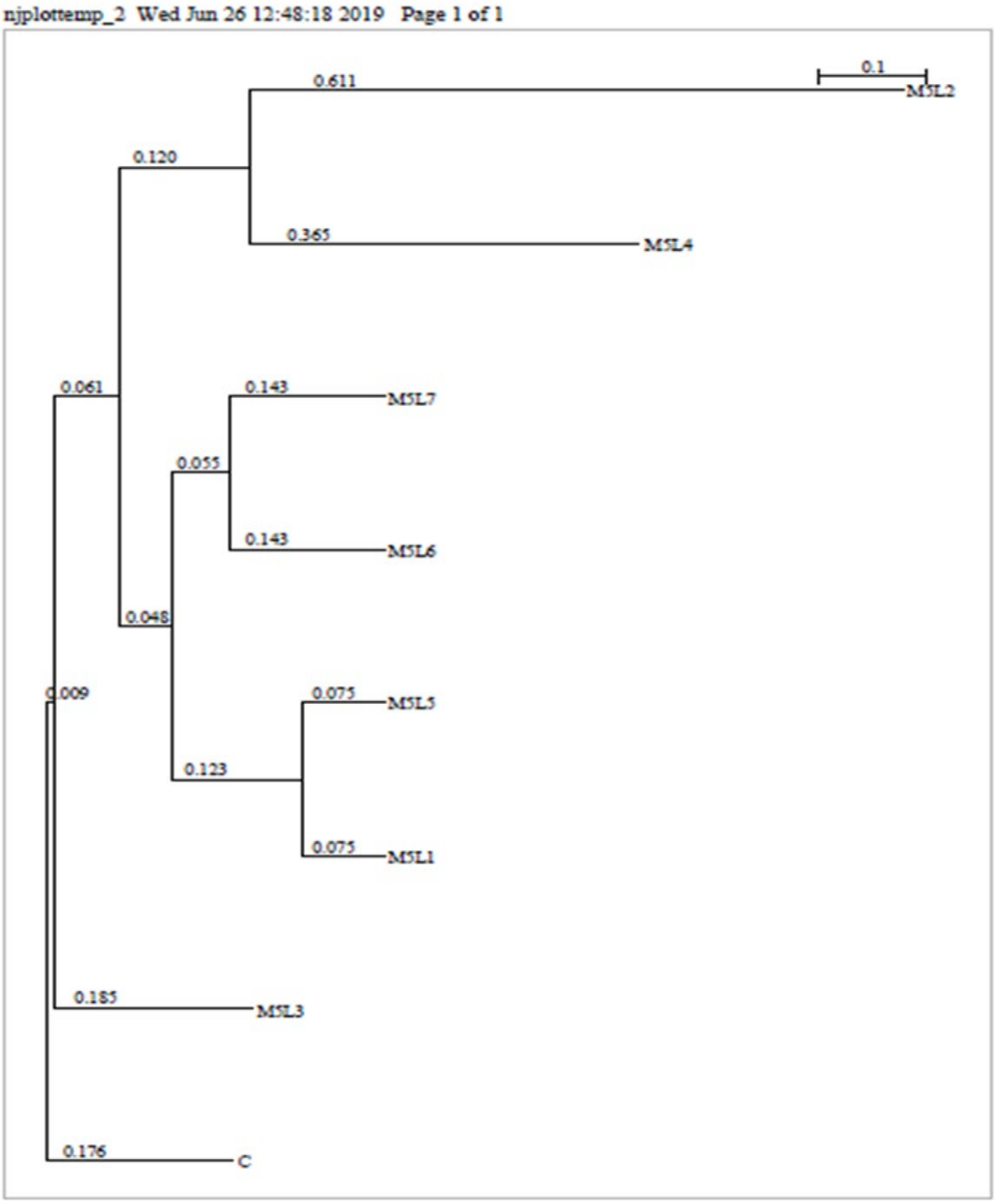

Figure 6.19: Phylogenetic tree of Mutant 7 lines with control of okra genotypes using RAPD markers.

N.B; [Mutant 5 Line 1- M5L1, Mutant 5 Line 2– M5L2, Mutant 5 Line 3– M5L3, Mutant 5 Line 4- M5L4, Mutant 5 Line 5– M5L5, Mutant 5 Line 6– M5L6, Mutant 5 Line 7– M5L7 and Control (Shamol Bangla) - C of Okra]

6.3.1.5 Genetic relationship among the mutant 7 lines with control of okra using SSR marker

6.3.1.5.1 PCR amplification

For the optimization of SSR amplification, different temperature profile and reaction components were tested. SSR bands are reproducible if the cycling parameters applied appropriately. PCR components were fixed as follows, 1µl (50 ng/µl) of template DNA, 0.5 µl (10 µM) of forward and reverse primer, 3 µl of nuclease-free water was mixed for 10 µl reaction volume. A denaturation temperature 95°C, annealing temperature of 55°C and an extension temperature at 72°C was found optimum for the SSR PCR amplification reaction.

6.3.1.5.2 Analysis of SSR profile

SSR bands obtained by different primers with mutant 7 lines with control genotypes of okra are shown in the Table 6.11. A total of 9 SSR primers were screened for the study of genetic diversity among the 7 lines okra genotypes and all of them gave scorable amplification. 16 SSR fragments were obtained by the 9 microsatellite markers. The data were further used for analysis.

The average number of bands per primer was 1.77 and out of 16 SSR bands, 14 of them were found as polymorphic (87.50%). The average number of polymorphic band per primer was 1.55. The average bands per primer was slightly lower than those studied with other workers (Schafleitner *et al*., 2013; Fougat *et al*., 2015; Sawadogo *et al*., 2009) with SSR markers, (Gulsen *et al*., 2007) with SRAP markers (Akash *et al*., 2013) with AFLP markers and (Prakash, 2011) with RAPD markers. Among all the 9 SSR markers tested least polymorphism was obtained by SSR 66 (50.00%) and highest polymorphism was obtained by SSR 9, SSR 52, SSR 54, SSR 56, SSR 63, SSR 78 and SSR 89 (100%). An average PIC value was calculated to be 0.106. Banding pattern and size range of the amplified product differ from each other. Amplified product varies from 100 bp-500 bp.

All the 16 SSR fragments were used for the analysis of genetic diversity among the individuals of okra. The cluster analysis (phylogenetic tree) showed a wide range of similarity ranging from 56.2 to 100% while 86 to 100% genetic similarity was

observed using Sequence-related amplified polymorphism (SRAP) markers in okra among 8 genotypes (Gulsen *et al*., 2007). Distance matrix indicates a narrow genetic base among different genotypes. High similarity among the accessions studied was expected because okra has highly self-pollinated species (Hamon & Koechlin *et al*., 1991) and similar results were also reported by Gulsen *et al*. (2007) and Sharma *et al*. (2015) who has also observed 100 % similarity in cultivars among the grapefruit cultivars. The diversity revealed in present study is equivalent to previous reports in okra (Gulsen *et al*., 2007; Yuan *et al*., 2014). However, Aladele *et al*. (2008) recorded lower genetic distance with RAPD in okra. A high genetic variability was also demonstrated by Torkpo *et al*. (2006).

Table 6.11: Microsatellite marker (SSR) used for the analysis of mutant okra genetic diversity

Sl. number	Primer code	Band Size	Number of Loci	Number of polymorphic loci		PIC
				Band number	Band frequency	
1	SSR 09	250	1	1	100.00	0.218
2	SSR 52	200-400	2	2	100.00	0.109
3	SSR 54	300	1	1	100.00	0
4	SSR 56	350	1	1	100.00	0
5	SSR 63	100-200	2	2	100.00	0
6	SSR 64	100-500	5	4	80.00	0.181
7	SSR 66	150-1200	2	1	50.00	0.234
8	SSR 78	300	1	1	100.00	0.218
9	SSR 89	350	1	1	100.00	0
Total			16	14	87.50	0.106

6.3.1.5.3 Banding pattern of different SSR primers

For the change in the nucleotide sequence or for the different PCR conditions, SSR profile was quite different for each primer. The detailed banding pattern and features of different SSR markers are described below:

Primer SSR 9

Primer SSR 9 gave 1 band in agarose gel electrophoresis and all of them were polymorphic. All the lines genotypes were amplified except line 2 with this primer. Fragment size was 250 bp. 100% polymorphism was found for this primer (Figure 6.20)

Primer SSR 52

2 bands were obtained by primer SSR 52 and 2 of them was polymorphic band. This marker gave clear SSR bands. 100% polymorphism was obtained by this primer. Fragments were found between 200 bp-400 bp (Figure 6.21).

Primer SSR 54

SSR-54 gave 1 band and the band was found polymorphic. 100% polymorphism was obtained and band was found 300 bp (Figure 6.22).

Primer SSR 56

SSR-56 gave only 1 band and intensity of the band was also poor. This primer gave 1 band and 1 of them was polymorphic band. Some of the genotypes were not amplified by this primer. Product size for this primer was 350 bp and polymorphism percentage was 100% (Figure 6.23).

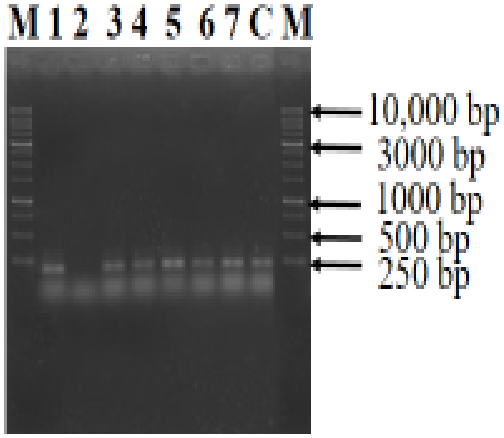

Figure 6.20: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 09

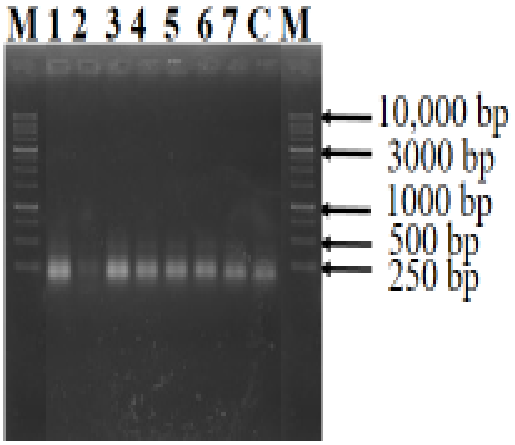

Figure 6.21: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 52

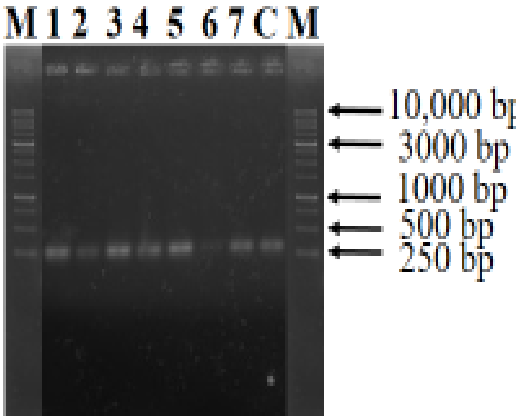

Figure 6.22: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 54

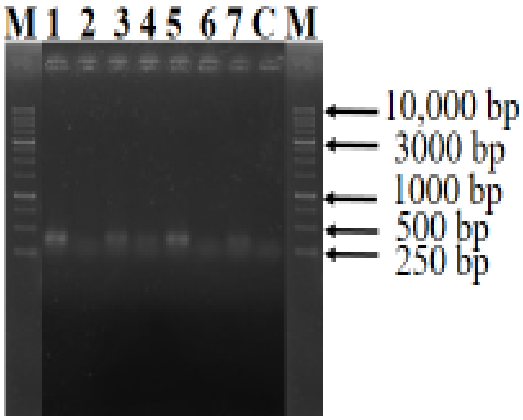

Figure 6.23: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 56

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

Primer SSR 63

SSR 63 obtained 2 bands and 2 of them were polymorphic bands. This marker gave most clear SSR bands in all lines genotypes. 100.00% polymorphism obtained by this primer. Fragments were found between 100 bp-200 bp (Figure 6.24).

Primer SSR 64

Highest number of bands was obtained by this primer and 5 bands were found. Total 5 bands were seen in this primer and 4 of them were found polymorphic. All the line genotypes gave good amplification with this primer. 80.00% polymorphism obtained by this primer. SSR fragments were found between 100 bp-500 bp in agarose gel (Figure 6.25).

Primer SSR 66

2 bands were obtained by primer SSR 66. Out of 2 bands, 1 band was polymorphic and polymorphism percentage was 50.00%. Fragments size ranges between 150 bp-1200 bp. Some bands were very sharp and found in line 1, 5 and 7 genotypes (Figure 6.26).

Primer SSR 78

1 band was obtained by this primer. One sharp band was obtained in all the line genotypes except line 2 with this primer. Polymorphism percentages were 100% and product size was found 300 bp (Figure 6.27).

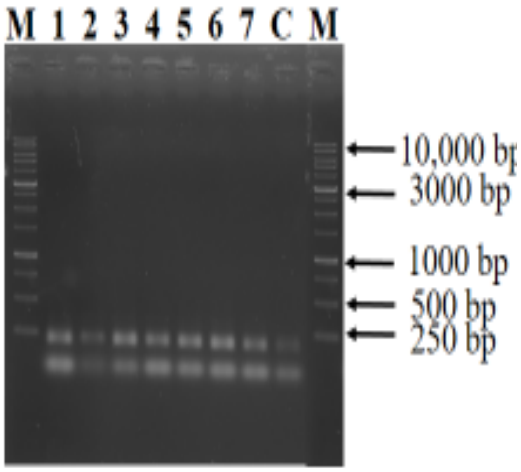

Figure 6.24: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 63

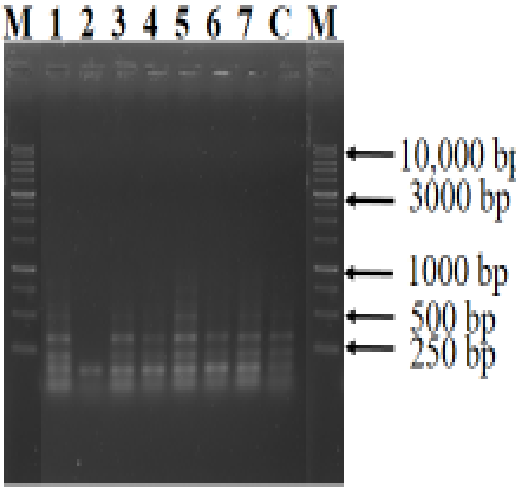

Figure 6.25: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 64

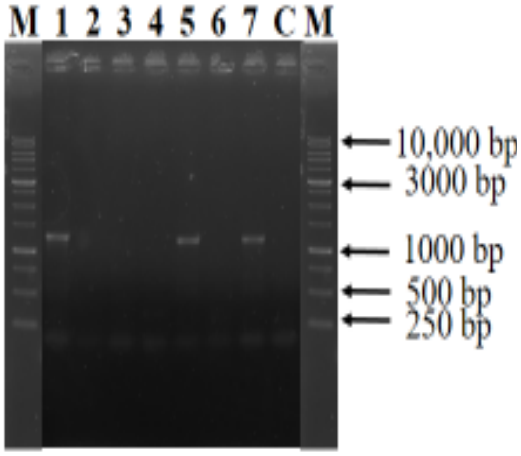

Figure 6.26: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 66

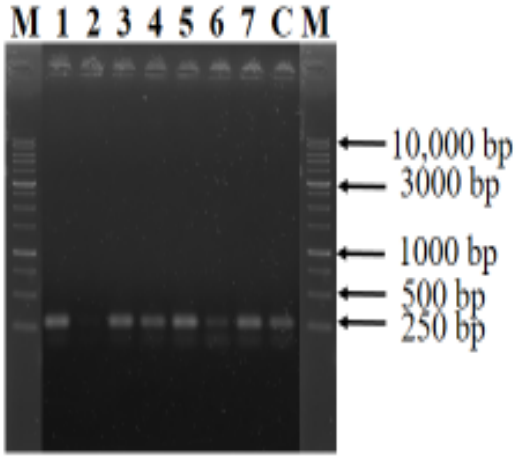

Figure 6.27: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 78

[N.B; **1**=Mutant 5 Line 1- M5L1, **2**=Mutant 5 Line 2– M5L2, **3**=Mutant 5 Line 3– M5L3, **4**=Mutant 5 Line 4- M5L4, **5**=Mutant 5 Line 5– M5L5, **6**=Mutant 5 Line 6– M5L6, **7**=Mutant 5 Line 7– M5L7, **C**=Control (Shamol Bangla) of Okra, **M**=Molecular Marker (1 kb DNA Ladder)].

Primer SSR 89

1 band was obtained by primer SSR 89. Out of 1 band, 1 band was polymorphic and polymorphism percentage was 100.00%. All the lines genotypes gave band. Fragments size was 350 bp (Figure 6.28).

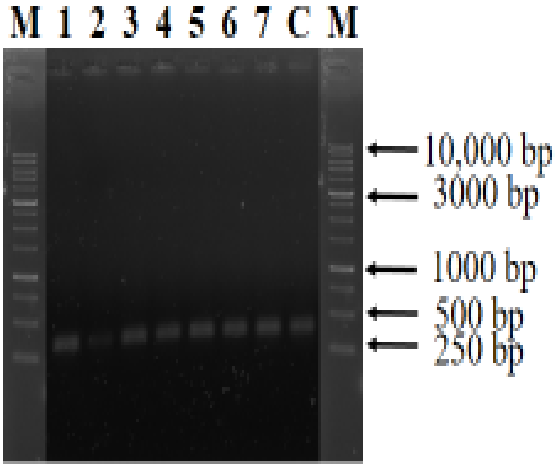

Figure 6.28: SSR profiles of Mutant 7 lines with control of okra genotypes using primer SSR 89

6.3.1.5.4 Phylogenetic tree analysis using SSR marker

For genetic diversity analysis, first a binary data matrix was constructed. SSR bands for all the primers were observed manually for detecting the presence or absence of bands for a particular locus. While manually inspecting the SSR gel images, the presence of the band was scored as 1 and absence of bands for particular locus was scored as 0. Thus a binary matrix was constructed for statistical analysis of the bands.

All the 16 SSR fragments were used for the analysis of genetic diversity among the individuals of mutant okra. Distance matrix indicates a narrow genetic base among different okra lines genotypes.

Statistical analysis of the banding pattern of all the microsatellite markers led to the development of a dendrogram on the basis of the genetic distances among different individuals of mutant okra. The dendrogram was prepared using an online software package called Dendro UPGMA and phylogenetic tree was done using the Jaccard index (Jaccard, 1908).

Phylogenetic rooted tree showed C to be very close to M5L3 and far from M5L2. On the other hand, M5L5, M5L7 & M5L1, M5L6 & M5L4 genetic relationship was found very close to each other (Figure 6.31).

	M5L1	M5L2	M5L3	M5L4	M5L5	M5L6	M5L7	C
M5L1	1	0.562	0.938	0.875	1	0.875	1	0.938
M5L2		1	0.6	0.643	0.562	0.643	0.562	0.6
M5L3			1	0.933	0.938	0.933	0.938	1
M5L4				1	0.875	1	0.875	0.933
M5L5					1	0.875	1	0.938
M5L6						1	0.875	0.933
M5L7							1	0.938
C								1

Figure 6.29: SSR similarity matrix of mutant 7 lines with control of okra.

	M5L1	M5L2	M5L3	M5L4	M5L5	M5L6	M5L7	C
M5L1	0	0.438	0.062	0.125	0	0.125	0	0.062
M5L2		0	0.4	0.357	0.438	0.357	0.438	0.4
M5L3			0	0.067	0.062	0.067	0.062	0
M5L4				0	0.125	0	0.125	0.067
M5L5					0	0.125	0	0.062
M5L6						0	0.125	0.067
M5L7							0	0.062
C								0

Figure 6.30: SSR distance matrix of mutant 7 lines with control of okra.

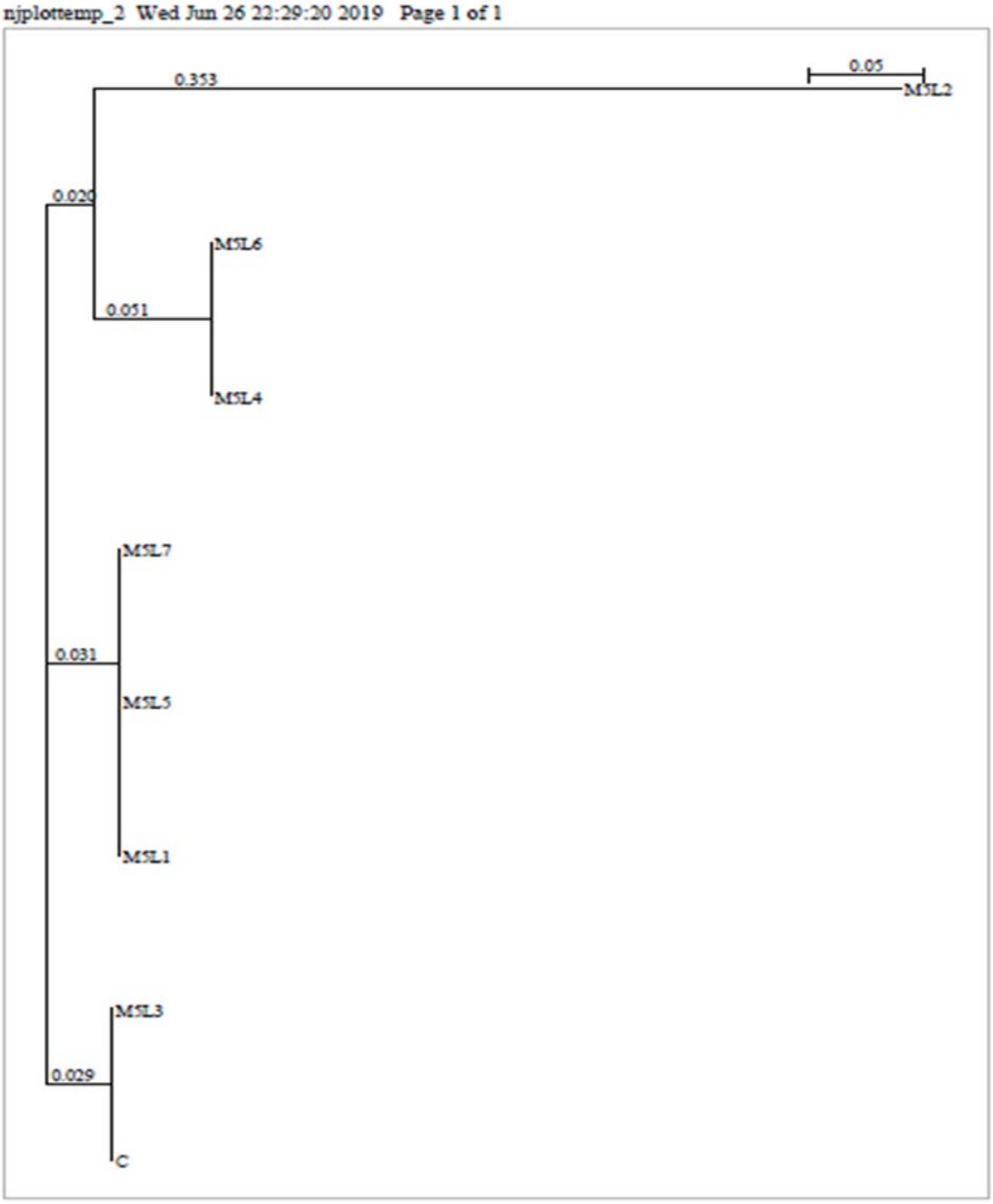

Figure 6.31: Phylogenetic tree of Mutant 7 lines with control of okra genotypes using SSR markers.

N.B; [Mutant 5 Line 1- M5L1, Mutant 5 Line 2– M5L2, Mutant 5 Line 3– M5L3, Mutant 5 Line 4- M5L4, Mutant 5 Line 5– M5L5, Mutant 5 Line 6– M5L6, Mutant 5 Line 7– M5L7 and Control (Shamol Bangla) - C of Okra].

6.3.1.6 Comparative analysis of RAPD and SSR marker

Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) marker were used for the genetic diversity analysis of mutant 7 lines with control of okra germplasm. The phylogenetic study has proved that there are some similarities in the diversity analysis. Same genomic DNA from 8 different individuals was subjected to PCR amplification by RAPD and SSR marker. A total of 20 RAPD primer and 9 pairs SSR primer were used for the present study.

Out of 20 random decamer primers, 10 gave scorable amplification with good polymorphism and 2 gave monomorphic (unique band) band (Table 6.10). 10 RAPD primers obtained 40 RAPD fragments of which 32 bands was found polymorphic. On the other hand, Out of 9 SSR primer tested, 9 gave good amplification and clear bands. The 9 SSR primers were able to produce a total of 16 SSR fragments and 14 of them were found polymorphic (Table 6.11). The average number of bands per RAPD primer was 4.0 and the average number of polymorphic band per RAPD primer was 3.2. On the other hand, average number of bands per SSR primer was 1.77 and average number of polymorphic band per SSR primer was 1.55. Therefore, the average number of bands per primer and polymorphism percentage of SSR marker were lower than RAPD marker. This is consistent with the previous study of 23 okra genotypes by Gulsen *et al*. (2007).

RAPD marker showed 80.00% polymorphism whereas SSR marker showed 87.50% polymorphism which was mostly similar among the 8 individuals of okra. Polymorphisms detected by AP-PCR or RAPD are inherited in a Mendelian man- ner and can be generated for any species without DNA sequence information (Welsh & Mc Clelland, 1990).

UPGMA dendrogram also showed several similar rooted tree clustering patterns for these two genetic markers. In RAPD, phylogenetic rooted tree showed C is very close to M5L3and far from M5L2. Also M5L5 & M5L1, M5L6 & M5L7, M5L4 & M5L2 were genetically in close relationship to each other (Figure 6.19). On the other hand in SSR, phylogenetic rooted tree showed C to be very close to M5L3 and far from M5L2. Also M5L5, M5L7 & M5L1, M5L6 & M5L4 genetic relationship was found very close to each other (Figure 6.31).

Both RAPD and SSR UPGMA cluster analysis showed 3 major branches. First branch, contains C genotype was found very close to M5L3 okra. Second branch, C genotype was found far distance from M5L2 okra. Third branch of other 5 genotypes showed mostly close relationship to each of other. It may be mentioned here that, in most of the cases, phylogenetic tree for RAPD and SSR markers showed similarities in morphological characteristics of the mutant okra lines.

Future experiments may be benefited by changing the experimental condition and overall method. Optimization of DNA extraction with other procedure may also be beneficial because DNA amount may vary with the different method. Very few works have been reported on mutant okra genetic diversity. Therefore, this study may give valuable information about okra genome. It was concluded that RAPD and SSR markers are efficient for understanding the genetic relationship among individuals, improving existing varieties, establishing a collection of germplasm, and involving markers into genetic linkage mapping. For example, during the harvesting season okra is harvested in every 3-4 days and use of pesticide in this period is very crucial for human health. Therefore development of disease resistant variety is a must. Yellow Vein Mosaic Virus and Aphids, for example, causes loss of yield and also interfere with the quality and structure of the fruit by altering the leaf and fruit structure of the plants (Panda & Khush, 1995). RAPD and SSR marker have the potentiality to develop new disease resistant variety using the genetic relationship of the existing genotypes by marker assisted selection. Therefore, these markers can be used in the okra breeding programs.

6.3.2 Unique Bands Identification

Among the PCR purified product of OPA- 9 and -11, OPA-11 showed good unique band sizes of 1300, 800, and 400 bp for M5L5 line (Figure 6.32 and 6.33).

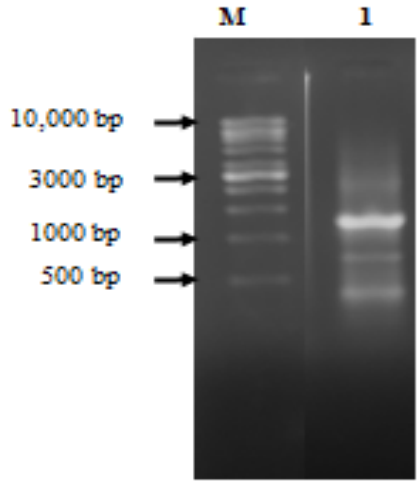

Figure 6.32: Band before purification (OPA 11)
N.B;[M= VC 1kb DNA ladder, 1=Sample A, B and C]

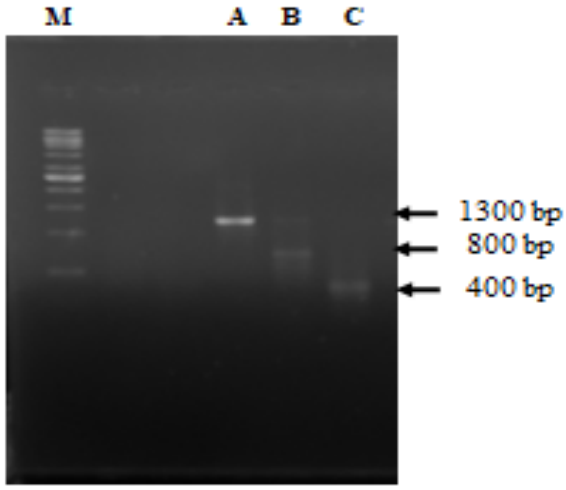

Figure 6.33: Band after purification (OPA 11)
(A-1300bp, B-800bp and C-400bp)

DNA sequencing of unique band of YVMV resistant with high yielding mutant line (M5L5) of okra using RAPD (OPA 11) marker was identified and given bellow (Figure 6.34):

Sample A.

AATGTATNNACTTGATTACTCGTCTCGATCATCACATGNGTAGCGATNAGGGTTGGATATGCGTCAC
ATTTGTCTGACTCTACTCTTACTNNNGCGGCATGTCTCCGGGGANCCGACTAGACGAGTCTTGTGN
NGAACTGTTACCAAAATCTTGCCAATTTCGACTGATGAAATTCACCCTGCTGATCACATNTNNNTA
CGTCGGTCAGGAGGACNTTATGTCTCTCAANNNCAATNNNNGACATGGTCTACNNNATACACTCTA
TAAGCTTAGCACGAAATGCTTGTCGATTGGCATCTTTGCTGATTTAAGATCGCGATGGATGGCGAAA
GGCAGCGGTATGCTCGAGGATATAGTCGACACCTCTAGACNNGNCCGACGAGATNNGCGCCCTTGCT
GACGACCGTCAGGTATCTCTGTCAGACAACTGATCGCGACGCATCANATGGNNGATTCGATGNTTAT
TGAGTCTAAATCACTCGTATNNGTACATGACTGTCTCCTTGACTNNGCTTGTGCTTACTTAAATTAC
CATTCTNNATGAACTCTTAAACTAATAACAACTAACCTTCGACACAATATCGGATTATTCGCACCTAC
TATGNAATTTTAAGATACAGTCACTTATACATTCATGATTATACGATTCATGAATGCTTGAAATCACC
ATAGGAGGAGAAGCATGTAACGGAATACCAGGTTCATGTGATGAACATGTGTTAAAACCTTCAACTC
GGCTAGAAATTCCTTCGACGCTTGCATATACATTTTCTTGATTGCCGCTTTCGGCAACCATCATCNN
GACTNAACATCATTNGATGCCCTGCTATACCCNTACTCTATAGCAACNNCTANNGAAAAGTTACNNT
ACGGTACGTCGNNTCGTAGTNCTGCGATGCAAANGGATCCAAATGCCTTCNTTGTTCCGATCTTATT
AACAAGGNGTNAAATTTTCAGTCGACTNGNCTAGCTGATTTTAGGAAAAAATTCCCNGAATNAGTC
GACCCCGGATNCCACTTTAGGCNTGGATGANNCCAAAAGTAAAAATNTCCAAATTTCGACTTCGGG
NNTACCTAACTAACCCTTTACCCTTTGGATTCTGATGGATCAGTCAATGGAAAT

Sample B.

GGCTGGGTGCGATTACTCCCTTAGGCGCGAATCGCGCGAGGATCTGAGTGAGAGGAGCACGGCTCCC
GATCGCCCCCATTCCGCGGTGCGTGTGCCGCGGGGACGCTGCGACTTGCTCGTGAATACTTCAAACG
ACTCTCGGCAACGGATATCTCGACTCTCGCTTCTCTGAAGAACGTGTGCTAATGCGATACTTGGTGT
GAATTGCCGAGTCCCATGCACCATCGAGTCTTTGATCGCGATTTGCGCCCCAAGCCGTTAGGCCAAG
GGTAAGTCTGCCTGGGTGTGACGAATCGTCGCCCCCCTCCAACACTTTTGCCGGAGGGAACGGGATG
CGTTGCGAGCGGACAATGGCCTCCCGTCCGCGCATCGACCGCGGTTGGTGCAAAATCGAGACATCGT
CATCCTGGGCGCCGCGACTATCGAAGGTAACGTTTCGGGCTGCCTCGATCGTTTCGCGCGCTACCGTC
GACCCCGGCTCTCCGACCCTTCCTGCACCGCGAACACGGTGCTCGAGGCGCGACCCCAGGTCAGGCG
GTACTACCCGCTGAGTTTAAGCATATCAATAATCGGAGGAAAACAAACTTACCAGGATTCGCTTACT
AACGGCTATTGTACAGGTCTACGGCGATTCATGATTATACGATTCATGAATGCTTGAAATCACCATAG
GAGGAGAAGCGAATAAGGGAATAGCAGGTTCAGGTGAATGA

Sample C.

GAAGCTCTCCCAGTACGTCGAGGTTTCCGTCCTCACTTCGCGGCCTCGGTCTAGCTTTGCATAAACAC
CATTCTCATACGTTCAGCATGGATTACTTAGTAAGAGGCGAGGATTCAAATCTATTTTACGTGTTATA
CCTGGACAGAACAGCGCGTCAGACGAGCTCGGTGTCACGGATCTCGAGTCCAATGAATGACAGTTCC
ATGAGGACCTTGAAACCAATGGCTTCGGACATGCCGAAATCACATATGATAACGATAGGGAGAGCAA
GGATGGCGATGGGACCGCAAAGGAGGCTTCCCGCTTCGACGGCGATTGAGACGCCTGGAGCAAACG
ACCAAAGAGAACATGGATGACGACGCCCCACGTAAATAAAGTCCTCTCCCTCTCCGCCTACGACGTG
GACTGCGATTGCTATGCATAAACTGTGATACTTTCGCCTCCTATCACTATTTACCATTGCTTGACCGG
GGTTTGACACGGGCAGGTAAGGATCCCCCGATGCATGAAATTGCTCACACCACACACCACCTAGCCA
TTTT

Figure 6.34: DNA sequence of unique bands of M5L5 okra using RAPD primer of OPA 11. N.B; [A-1300bp, B-800bp and C-400bp]

From the sequence analysis it can be concluded that-

A. I got the partial sequence but I could not identifiy the gene.

B. I got partial sequence similarity with Internal Transcribed Spaces (ITS) ribosomal DNA of okra and it may be responsible for mutation. As a result, the ITS gene effect may be showed to have the resistance to YVMV and the high yielding potential.

C. I got partial sequence similarities to Glycerol-3- phosphate Acyl transferage. It is responsible for cold/chilling tolerance gene.

6.4 Summary

Okra is considered as the most important vegetable all over the world. An attempt was taken to study the molecular characterization (genetic diversity) among the mutant 7 lines with control and identification of unique bands by gene sequence of okra in Bangladesh. This study was undertaken to evaluate the molecular characterization of the YVMV resistant mutant lines by the development of molecular markers such as RAPD and SSR and finally it identified that the YVMV resistant gene in mutant okra lines was the first among the molecular fingerprinting of mutant okra genotypes collected from the cultivated field of Rajshahi University campus Bangladesh.

Molecular characterization of nutrient rich and YVMV resistant mutant okra lines was performed by using RAPD and SSR markers. Genomic DNA (gDNA) of 7 mutant okra lines was extracted and purified by modified CTAB method, then amplified by PCR; RAPD and SSR bands were analyzed. Data were used for construction of phylogenetic tree by Dendrogram using UPGMA with Jaccard index of bioinformatics. Unique RAPD bands were cut and purified by DNA kit. YVMV resistant mutant okra Genes were Sequenced and identified by 1st BASE Co, Malaysia.

Molecular characteristics of M5 okra lines such as M5L1, M5L2, M5L3, M5L4, M5L5, M5L6, M5L7 were compared with control C to study the genetic diversity using RAPD and SSR markers. gDNA was extracted and subjected to PCR amplification using 20 RAPD and 9 SSR primers. Out of 20 RAPD markers, 10 gave good amplification and scorable polymorphism and 2 gave monomorphic (unique band) band. A total of 40 RAPD fragments were formed by the 10 RAPD primers.

Out of 40 bands, 32 were polymorphic. About 80% polymorphism was found among the 7 lines. Average number of bands per primer was 4.0 and average number of polymorphic bands per primer was 3.2. Average PIC value was calculated to be 0.415. Phylogenetic rooted tree showed C is very close to M5L3and far from M5L2. On the other hand, M5L5 & M5L1, M5L6 & M5L7, M5L4 & M5L2 were found genetically in close relationship to each other. A total of 9 SSR primers were screened and all of them gave scorable amplification. The 9 SSR primers gave 16 bands and 14 of them were polymorphic. Number of bands per primer was calculated to be 1.77 and the number of polymorphic band per primer was 1.55. About.87.5% polymorphism was observed among individuals of okra lines. An average PIC value was 0.106. Phylogenetic tree showed C to be very close to M5L3 and far from M5L2. On the other hand, M5L5, M5L7 & M5L1, M5L6 & M5L4 genetic relationship was found very close to each other. It may be mentioned here that, in most of the cases, phylogenetic tree for RAPD and SSR markers showed similarities in morphological characteristics of the mutant okra lines. Among OPA- 9 and -11, OPA-11 showed unique band sizes of 1300, 800, and 400 bp for M5L5 line. Partial sequence homology of 800 bp with Internal Transcribed Spaces (ITS) ribosomal DNA of okra was observed that might be responsible for demonstrating the mutated phenotype (YVMV resistance and high yield) of M5L5 okra.

CHAPTER SEVEN
REINVESTIGATION OF NUTRITIONAL AND CYTOTOXIC LEVEL OF MUTANT M6 OKRA

7.1 Introduction

To determine and establish nutrient enriched mutant okra, reconfirmation study of nutrient is necessary. On the other hand, cytotoxicity expresses their level of toxicity to use the pod as easily edible for human without any hazards. Also the materials usefulness will be clarified for traditional medicine. Therefore, Nutritional (Qualitative and Quantitative) properties and Cytotoxicity were re-investigated on Mutant M6 plants.

7.2 Materials and Methods

The mutant M6 plants of okra pod sample were collected from the cultivated field of Biochemistry Research Garden. Qualitative and quantitative analyses of nutritional content and cytotoxicity effects were done by the methodology described before (Chapter 4).

7.3 Results and Discussion

7.3.1 Qualitative analysis of phytochemicals

Abelmoschus esculentus premature pods contain most of the phytoconstituent that are responsible for their anti-oxidative activities. Table 7.1 shows the phytochemical composition of mutant 7 lines with control of okra premature pod methanolic extract.

Table 7.1: The qualitative phytochemical investigation of methanolic extract of mutant 7 lines with control of *A. esculentus*

Name of the test	**Methanolic extract result of-**							
	M6L1	M6L2	M6L3	M6L4	M6L5	M6L6	M6L7	C
Alkaloids:								
Dragendorff's test	+	-	-	+	+	-	-	+
Hager's test	-	-	+	+	+	-	+	+
Wagner's test	Br+	-	Br+	-	Br+	Br+	-	Br+
Mayer's test	+	+	-	-	+	+	-	+
Carbohydrates:								
Anthrone test	+	-	-	+	+	-	-	+
Benedict's test	-	+	+	-	+	+	+	+
Fehling's test	+	-	-	+	+	-	-	+
Molisch's test	-	-	+	-	+	-	+	+
Flavonoids:								
Shinoda's test	+	+	+	+	+	+	+	+
Glycosides:								
Molisch's test	+	+	+	+	+	+	+	+
Triterpenoids:								
Liebermann-Burchard	-	+	-	-	-	+	-	-
Resins:	+	+	+	+	+	+	+	+
Saponins:	+	+	+	+	+	+	+	+
Steroids:								
Liebermann-Burchard	Gr+	-	-	Gr+	Gr+	-	-	Gr+
Salkowski reaction	-	-	-	-	-	-	-	-
Tannins:	Br+	Gr+	Br+	Gr+	Gr+	Br+	Gr+	Gr+

[**Note:** "+" indicates the presence of the relevant phytoconstituents, whereas "–" indicates the absence, Br=Brown, Gr=Green of methanol extract of mutant 7 lines with control of *A. esculentus* pod respectively].

From the above qualitative analysis of phytochemicals, it was observed that in most of the cases mutant lines showed similarities to each of other and M6L5 line was found same as Control (SB). In this study, the qualitative phytochemical analysis of pods

methanolic crude extracts of mutant 7 lines with control of okra reconfirmed the presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins. So, this plant might be right choice to serve the therapeutic purposes.

7.3.2 Quantitative analysis of nutritional properties

The nutritional properties are given in the following Table 7.2:

Table 7.2: Nutritional content of nutrient rich (SB-present and previous study values) and nutrient enriched mutant (Mutant 7 lines) new variety of *A. esculentus* premature pods.

Status of Study	Name of Lines/Variety	g% (approx..)			mg/100g (approx..)
		Carbohydrate	Protein	Lipid	Vitamin-C
Present Chapter Study	Control (SB)	7.731	2.30	0.10	25.73
	M6L1	8.082	2.93	**0.15**	26.59
	M6L2	6.935	2.11	0.10	24.64
	M6L3	7.582	2.72	0.12	25.15
	M6L4	7.226	2.52	0.08	24.82
	M6L5	**8.643**	**2.97**	0.11	**29.62**
	M6L6	7.839	2.55	0.14	25.94
	M6L7	7.947	2.73	0.12	27.53
Previous Chapter Study	SB	7.42	2.15	0.09	23.95

From the comparative study of the above Table (7.2), the present study showed the highest nutrient values (nutrient rich) in all content of all lines in comparison with the previous study (Chapter 4) of SB values. On the other hand, out of 4 nutritional parameters 3 parameters showed highest in M6L5 line such as: Carbohydrate 8.643 g%, protein 2.97 g% and vitamin-C 29.62 (mg/100g). In most of the cases, mutant okra values also showed higher performance than control (SB). Therefore, Mutant okra 7 lines were reconfirmed as the nutrient rich variety resistant to YVMV.

7.3.3 Brian Shrimp Lethality Bioassay (Cytotoxicity effect)

The Brian Shrimp Lethality Bioassay LC_{50} values are given in the following Table:

Table 7.3: LC_{50} value for mutant 7 lines with control extracts of *A. esculentus*

Conc. µg/ml	Log conc.	% of mortality									LC_{50} (µg/ml)								
		Control (SB)	M6L1	M6L2	M6L3	M6L4	M6L5	M6L6	M6L7	GA	Control (SB)	M6L1	M6L2	M6L3	M6L4	M6L5	M6L6	M6L7	GA
10	0.99	0	0	0	0	0	0	0	0	46.66	890.74	**842.62**	1335.69	1034.53	1135.23	928.43	1035.83	934.58	**13.45**
100	1.99	16.66	23.33	20	20	20	23.33	13.33	16.66	70									
200	2.30	36.66	33.33	23.33	33.33	30	33.33	30	33.33	86.66									
400	2.60	36.66	36.66	33.33	33.33	30	33.33	36.66	36.66	100									
800	2.90	43.33	46.66	36.66	40	40	43.33	36.66	40	100									

The lethality of the methanolic extracts of mutant 7 lines with control premature pod of *A. esculentus* to brine shrimp was determined after 24 hours of exposure to the test solutions and the positive control, gallic acid using the method of Meyer *et al*. (1982). The median lethal concentration (LC_{50}) of brine shrimp lethality was obtained from probit statistical analysis. In this bioassay, the mortality rate of brine shrimp was found to increase with the increase in concentration of the test sample. So it was observed that the extracts exhibited brine shrimp having low cytotoxicity (mild/slightly toxic) in a dose dependent manner (Table 7.3). The value of LC_{50}, indicates the low cytotoxic effect of okra pod extracts. Extracts lowest LC_{50} value of brine shrimp lethality was found in M6L1 842.62 µg/ml among the of mutant 7 lines with control of okra and Standard cytotoxic agent gallic acid was found to exhibit higher cytotoxicity with a lower LC_{50} value 13.45 µg/ml, maximum mortalities GA (100%) were observed at a concentration of 400 µg/ml. Extracts highest LC_{50} value of brine shrimp lethality was found in M6L2 1335.69 among the mutant lines of okra. The lethality activity (LC_{50}) of different lines extract was in the order of M6L1 (842.62) < C (890.74) < M6L5 (928.43) < M6L7 (934.58) < M6L3 (1034.53) < M6L6 (1035.83) < M6L4 (1135.23) < M6L2 (1335.69) (Table 7.3). Above range value was showed consistent as potent for some varieties and some varieties inconsistency was also found among the mutant 7 lines with control premature pod extract of *A. esculentus* compared to the other reported values. Gupta *et al*. (1996) reported that the LC_{50} of 1000 µg/ml was estimated as potent (active). Another report was noticed by Meyer *et al*. (1982) that the LC_{50} value of less than 1000 µg/ml is toxic while LC_{50} value of greater than 1000 µg/ml is non-toxic. Olowa & Nuñeza *et al*. (2013) reported that the extracts of *L. camara*, *C. odorata*, and *E. hirta* were potent against the brine shrimp with LC_{50} values of 55, 10, and 100 ppm (µg/ml), respectively. Ahmed & Jahan (2017) reported that the brine shrimp lethality assay of the extract of *Xylaria hypoxylon* demonstrated medium cytotoxicity with LC_{50} value 327.00 µg/ml after 6 hours. However, in this study the extracts of most of the lines (C-SB, M6L1, M6L5 and M6L7) were showed slightly toxic and some varieties (M6L2, M6L3, M6L4 and M6L6) showed non-toxic range values among the 10 varieties of okra. From the study of LC_{50} values, present control (SB) value 890.74 is less than M6L5 value 928.43 but previous study control (SB) value 934.57 was little bit higher (Table 4.7) than the

M6L5 value. So, as the best line M6L5 compounds might be responsible near the same bioactivity to control. Other way, line M6L5 showed higher value 928.43 than M6L1 value 842.62 but lower than M6L7 value 934.58. Therefore, in phylogenetic tree and morphological characteristics analysis M6L5 was found having similarities and close relationship with M6L7 and M6L1. It is noted that the rich (higher value) on morphology, nutritional and Molecular analysis the M6L5 line also showed the slightly toxic LC_{50} value 928.43 µg/ml. It may be a good source for production of new drugs for various complications among the mutant 7 lines with control premature pod extract of *A. esculentus*. For some lines values inconsistency, further study is needed for more accuracy in future. Finally, it can be concluded that the methanolic extracts of mutant 7 lines with control premature pod of okra tested showed a little bit (mild toxic/slightly toxic) toxicity against brine shrimp larvicidal activity (Table 7.3). Therefore, the uses of this plant species in traditional medicine and pod can be used as edible fruit without hazard. The result acquired from the brine shrimp lethality bioassay of premature pod methanolic extracts of mutant 7 lines of *A. esculentus* can be utilized as a preliminary guide for the isolation of cytotoxic compounds.

7.4 Summary

The qualitative phytochemical analysis of pods methanolic crude extracts of mutant 7 lines with control of okra reconfirmed the presence of various phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins. So, this plant might be right choice to serve the therapeutic purposes. In quantitative analysis, out of 4 nutritional parameters 3 parameters were showed highest in M6L5 line such as: Carbohydrate 8.643 g%, protein 2.97 g% and vitamin-C 29.62 (mg/100g). Therefore, nutrient rich variety was established from the study of mutant M6 plants, especially line M6L5.

In BSLT, it was observed and reconfirmed that the extracts exhibited brine shrimp low cytotoxicity (mild/slightly toxic) in a dose dependent manner (Table 7.3). The value of LC_{50}, indicates the low cytotoxic effect of okra pod extracts. Extracts lowest LC_{50} value of brine shrimp lethality was found in M6L1 842.62 µg/ml among the of mutant 7 lines with control of okra and Standard cytotoxic agent gallic acid was found to exhibit higher cytotoxicity with a lower LC_{50} value 13.45 µg/ml, maximum

mortalities GA (100%) were observed at a concentration of 400 μg/ml. In the extracts the highest LC_{50} value of brine shrimp lethality was found in M6L2 1335.69 among the mutant lines of okra. The lethality activity (LC_{50}) of different lines extract was in the order of M6L1 (842.62) < C (890.74) < M6L5 (928.43) < M6L7 (934.58) < M6L3 (1034.53) < M6L6 (1035.83) < M6L4 (1135.23) < M6L2 (1335.69) (Table 7.3).

M6L5 line revealed itself as the nutrient rich (higher nutrient contents) best line and no significant change was observed with control compared to the previous (chapter 4) cytotoxic study to health issue.

CHAPTER EIGHT
CONCLUSION

- This study has successfully identified the Shamol Bangla as the best variety among the cultivated 10 varieties based on field performance against YVMV, nutrient contents, phytochemicals and medicinal values.
- Genetic improvement of nutrient rich (SB) variety of okra through induced mutation technology for resistance to YVMV successfully achieved 7 lines of mutant okra (from the generation M1 to M6 plants) as the developed new okra variety.
- Molecular characterization of mutant okra 7 lines (M5) revealed genetic relationship to each of other and identified high yielding and YVMV resistant gene in mutant okra line (M5L5). The study identified that partial gene sequences (M5L5) of molecular markers provide the knowledge about the mutation in the marker genes and might be used for screening of YVMV resistant okra lines or varieties.
- Advanced okra 7 lines (M6) re-investigated, subject to analyse nutritional (qualitative and quantitative) content and cytotoxic level, showed the higher nutrient content in M6L5 line as the nutrient rich best line and no significant change was observed with control compared to the previous (chapter 4) study related to health issue.

The Future line of work

- This study already characterized the 5^{th} (fifth) generation of advanced lines which may be back crossed to identify the QTL or responsible gene for the resistance to YVMV disease and characterized the 6^{th} (sixth) generation of advanced lines which might be released as improved varieties in terms of YVMV resistance in future after successful field trials under seed certification authority.

- To emphasize the narrow genetic diversity of okra a large number of RAPD and SSR markers should be used to for achieving much more coverage of the okra genome.
- SSR and RAPD markers were found potential to amplify the conserved regions of okra. Other molecular markers such as Inter-Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP) and other markers should also be investigated for the enhancement of okra breeding program.
- Molecular characterization study of mutant okra lines will provide an opportunity for plant breeders to develop new and improved cultivars with desirable characteristics.

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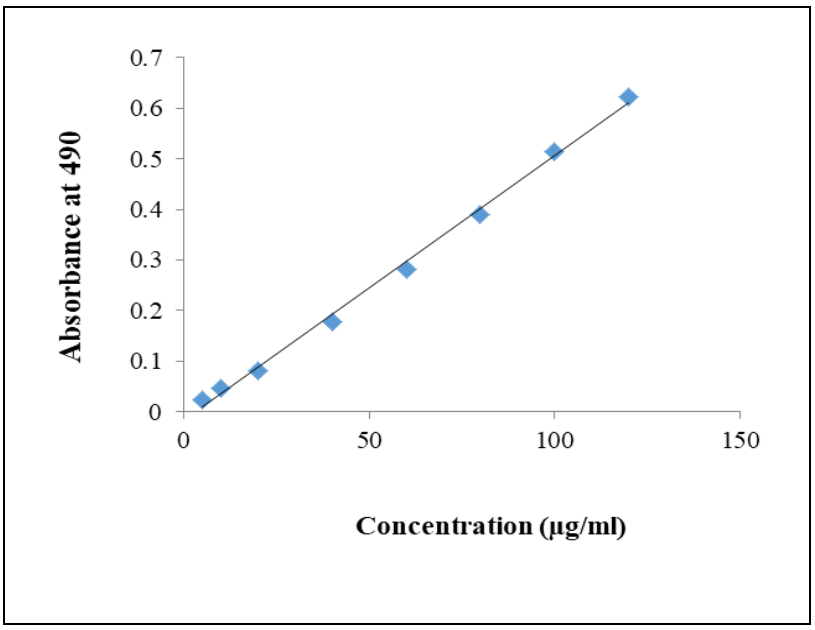
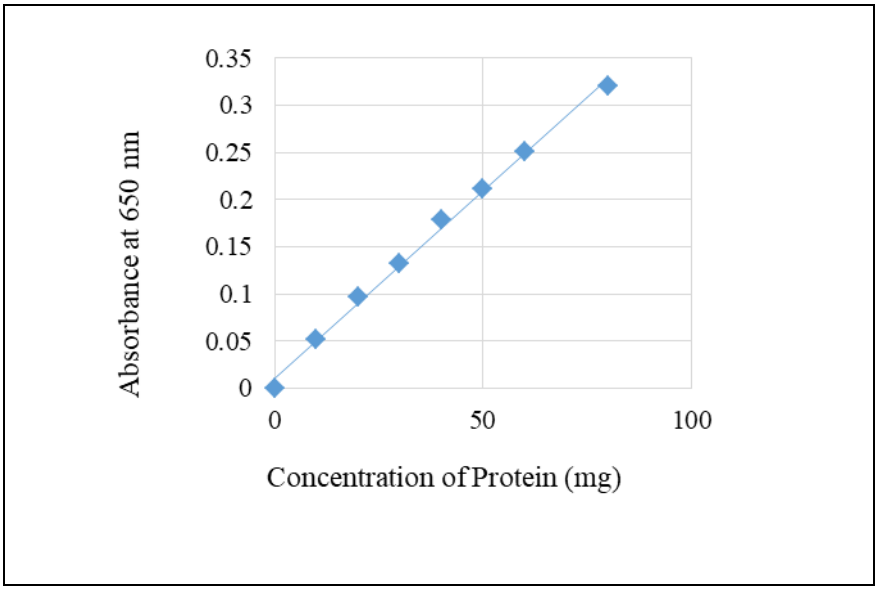
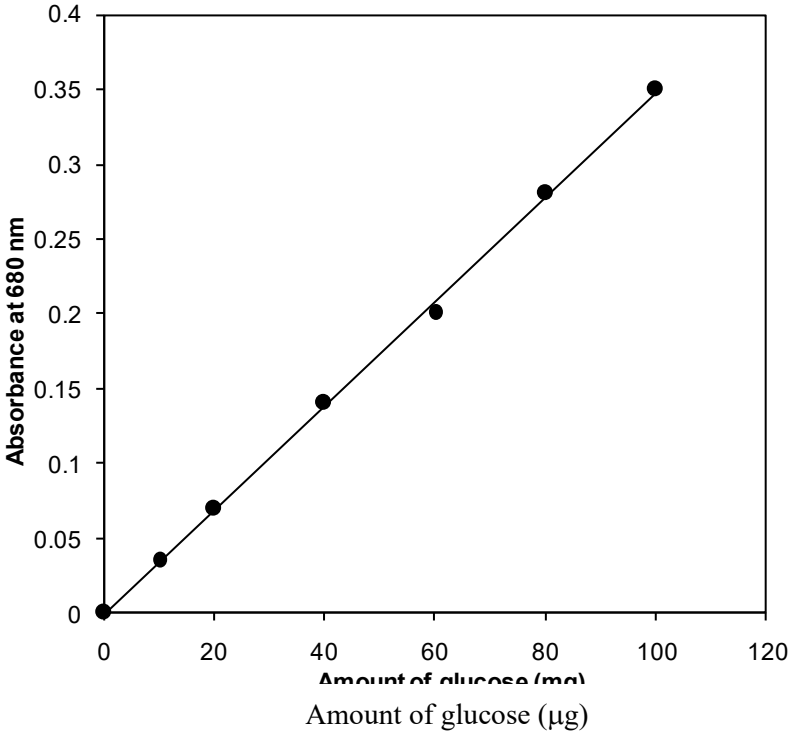
10 Appendices



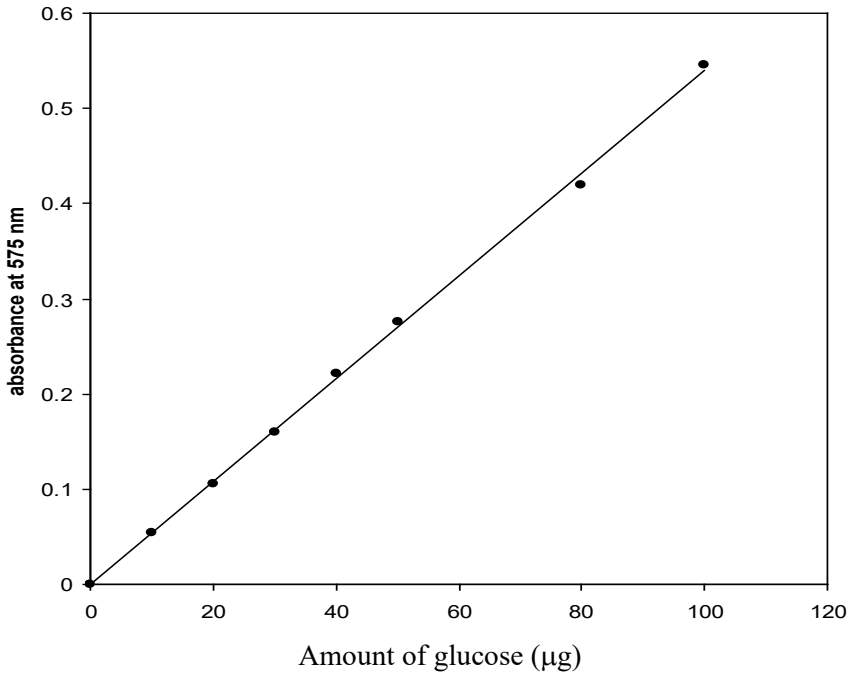
Appendix 10.1: Standard curve of glucose for the determination of carbohydrate.



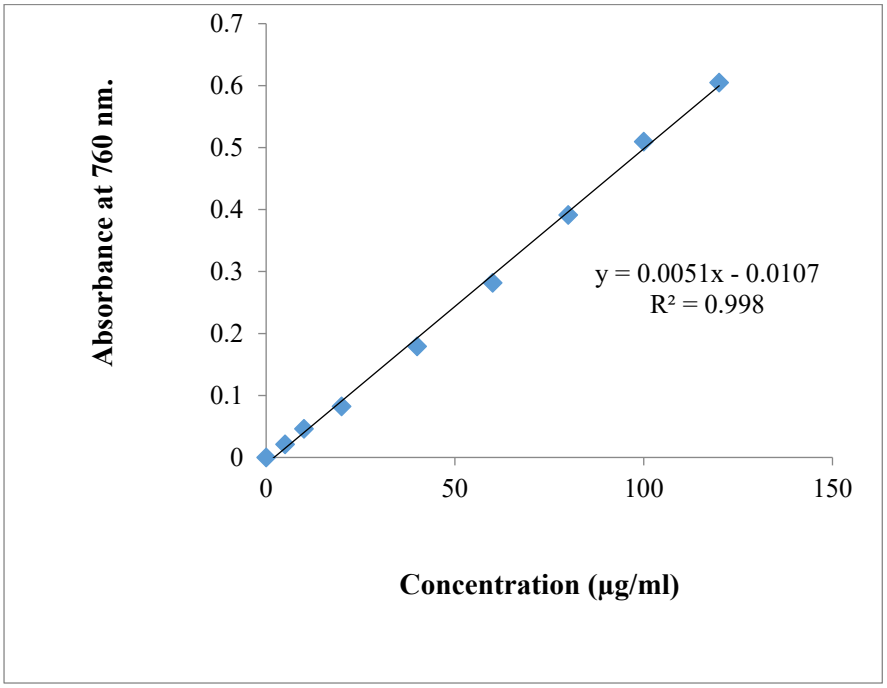
Appendix 10.2: Standard curve for the determination of protein concentration by Lowry method.



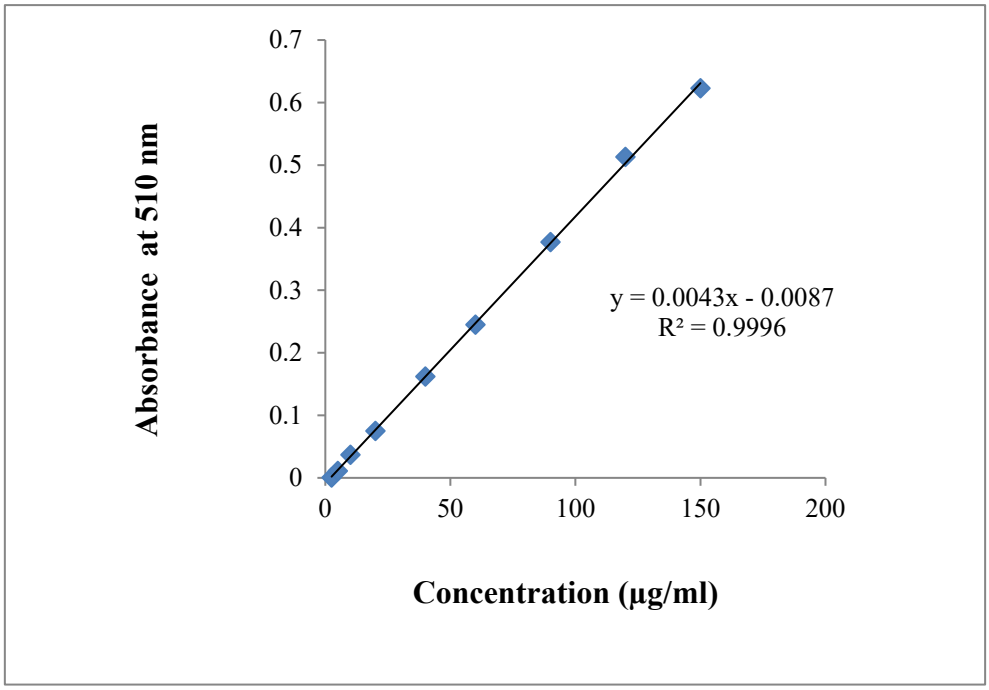
Appendix 10.3: Standard curve of glucose for estimation of total soluble sugar.



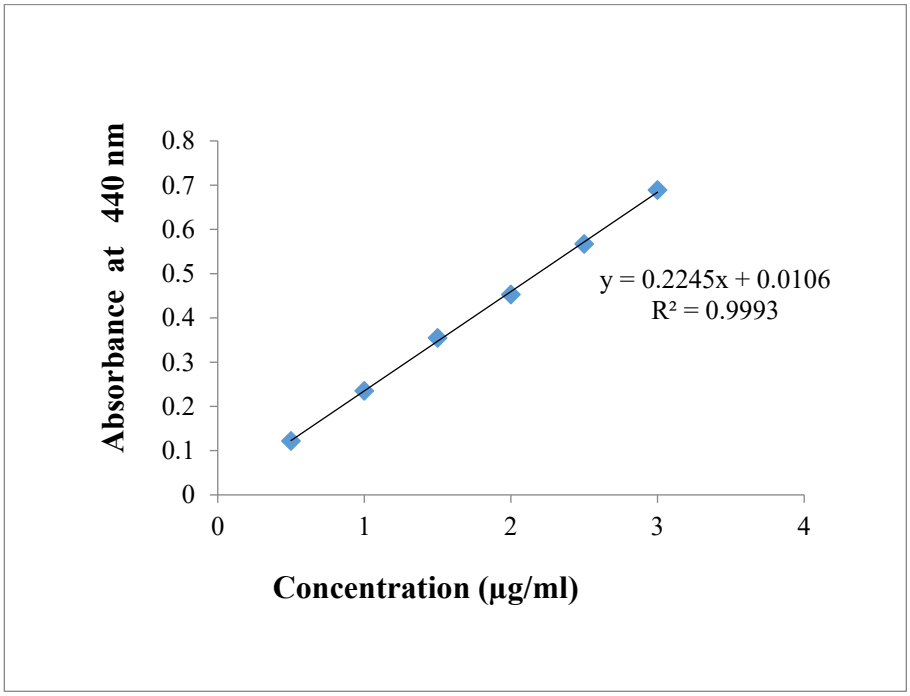
Appendix 10.4: Standard curve of glucose for estimation of reducing sugar.



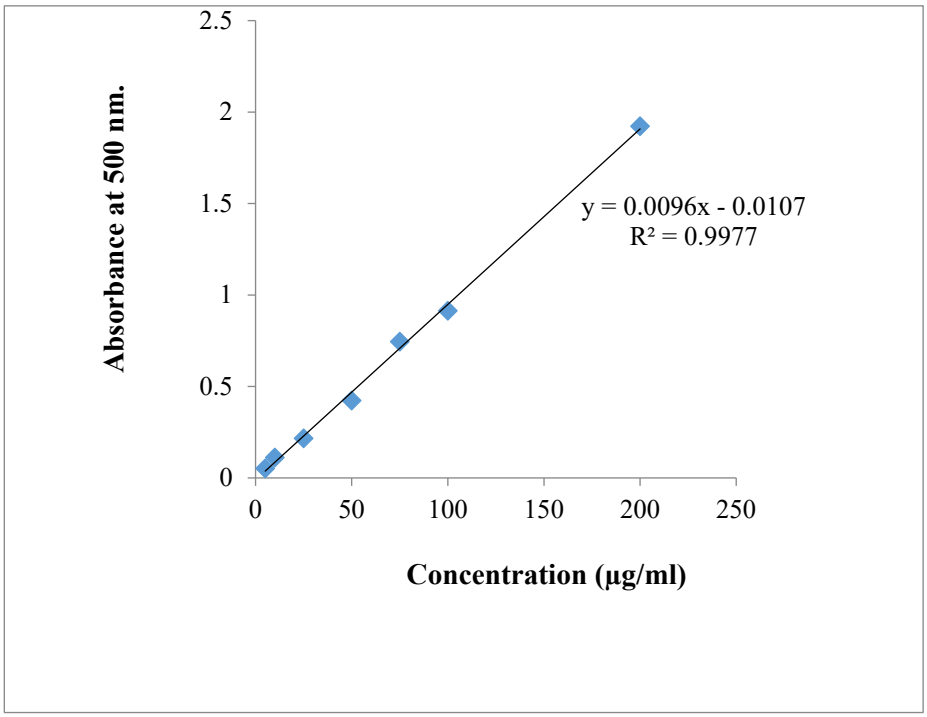
Appendix 10.5: Standard curve of Gallic Acid (GAE) for the determination of total phenolics content.



Appendix 10.6: Standard curve of Catechin (CAT) for the determination of total flavonoids content.



Appendix 10.7: Standard curve of Quercetine (QU) for the determination of total flavonols content.



Appendix 10.8: Standard curve of Catechin (CAT) for the determination of total proanthocyanidins content.