BIOLOGY, PHYTOCHEMISTRY AND *IN VITRO* PROPAGATION OF *CITRUS MACROPTERA* MONT. VAR. *ANNAMENSIS* (SATKARA)

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Registration No. FH/50, Session: 2007-2008

For

Degree of Doctor of Philosophy

Faculty of Biological Sciences
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January, 2012

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Dedicated to my beloved

Father: Md. Abul Hussain

and

Mother: Mrs. Shofikunnessa

DECLARATION

I do hereby declare that the work presented in the thesis entitled "Biology, Phytochemistry and *in vitro* propagation of *Citrus macroptera* Mont.Var. *annamensis* (Satkara)." was carried out by me under the supervision of (1) Professor Dr. Syed Hadiuzzaman, Supervisor (2) Professor Dr. Sitesh Chandra Bachar, Joint Suprvisor and (3) Dr. Md Azmat Ullah, PSO, Joint Suprvisor. This thesis or any part of it has not been submitted in any form to other institution for any other degree.

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CERTIFICATE

This is to certify that the thesis entitled "Biology, Phytochemistry and *in vitro* propagation of *Citrus macroptera* Montr.Var. *annamensis* (Satkara)" is a record of original research carried out at Department of Botany and Department of Pharmaceutical Technology of Dhaka University and Citrus Research Center, Bangladesh Agricultural Research Institute (BARI), Jaintapur, Sylhet under our joint supervision. We examined thoroughly the present work and found it original. The sources of literatures are duly acknowledged. It is further certified that this thesis is suitable for submission for the award of Ph.D. degree.

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Acknowledgement

Starting in the name of Almighty Allah, the great and merciful, His help supported me to complete this work. The thesis is the result of almost 36 month works whereby I have been accompanied and supported by many people from home and abroad. It is a pleasant aspect that I have now the opportunity to express my gratitude to all of them.

The first and foremost person I would like to thank my principal supervisor, Dr. Syed Hadiuzzaman, Supernumerary Professor, Department of Botany, University of Dhaka, for his initial inspiration and continuous guidance throughout the whole work. I am indebted to him more than he knows. He supported me for my personal health, family co-operation as a dedicating guardian and above all every aspect of my works. Without his encouragement, suggestions and co-operation it would be impossible for me to accomplish this work.

I am also grateful to my joint supervisor Dr. Sitesh Chandra Bachar, Professor, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka for his proper and enthusiastic guidance and assistance to complete the phytochemical part of the works. He was so cordial and cooperative with friendly mentality. For that I could not feel any pressure rather enjoyed his company.

I express my heartfelt gratitude to my another joint supervisor Dr. Md Azmat Ullah, Principal Scientific Officer, Pomology Division, Horticultural Center, Bangladesh Agricultural Research Institute (BARI), Gazipur for his dedicating guidance to complete the biological part. He also inspired me from very begining to do the works on this crop. He spent more time for me from plant selection to Institutional support.

I am grateful to the Chairman, Department of Botany, University of Dhaka for providing me scope of study in this Department as a registered Ph.D. student of University of Dhaka. He provided me all logistic supports wherever necessary for my works.

I express my gratitude to the University Grant Commission (UGC) of Bangladesh for providing me the scholarship throughout my works. I am also thankful to Directorate of Secondary and Higher Education, Ministry of Education for granting me full time deputation to complete the work smoothly.

My profound gratitude to Professor Dr. Md Abul Hassan, Professor Dr. Tahsina Rahim, Professor Dr. Md Abul Bashar, Professor Dr. Md Imdadul Haque, Professor Dr. Shamimul Alam and Professor Dr. Mihir Lal Saha of Department of Botany, University of Dhaka for their constant encouragement and help during my work.

It is my great pleasure to express my indebtedness to Dr Md. Khalilur Rahaman, PSO, Dr. Shah Md. Lutfur Rahaman, S.S.O, and Mr. Anwar Hussain, S.O. of Citrus Research Station, BARI, Jaintapur, Sylhet for providing me research facilities in citrus garden and valuable field experience throughout the works.

I am deeply indebted to Mr. Anis Jahangir, Director Research, Dr Sahina Islam, PSO, Mrs. Ferdous Ara, P.S.O, Mrs. Konika Mondal, S.S.O, Mr. Moniruzzaman Khandakar, S.O, of BCSIR Laboratories, Dr. Kudret-E-Kuda Road, Dhanmondi, Dhaka for providing me the scope of Laboratory use, valuable suggestion and their guidance.

I have great pleasure to thank to Dr. Satyajit Dey Sarker, Dr. Lutfun Nahar, Department of Pharmacy, School of applied Sciences, University of Wolverhampton, West Midland, UK for biochemical analysis of the essential oil of Satkara sent from Bangladesh and their valuable guidance.

I am also indebted to Dr. Abu Turab Muhammad Rahim, Professor, Nutrition Science of Dhaka University for his help and logistic support during nutrient analysis of the fruits. My sincere thanks to Mrs. Rumana, S.O, National Herbarium and Ph.D. fellow, Mrs. Nilufar Yasmin, lecturer in botany and Ph.D. fellow of Department of Botany, Md. Ashraful Islam, MSc Student of Deparment of Chemistry, University of Dhaka for their technical supports in different experiment of this work.

Sincerely I owe my indebtedness to my classmate Mr. Rupan Kanti Sheel, Joint Secretary, Ministry of Primary and Mass Education, close friend and ex- colleague Mr. Tahiat Hussain, Deputy Secretary, Ministry of Education for their administrative support, otherwise it would be a tough job for me to get leave and grant.

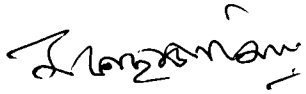
I express my heartiest indebtedness to my father Md. Abul Hussain and my mother Mrs. Shofikunnessa for their constant encouragement, love, dua and financial supports. My two sisters Sara and Lipi, cousin brother Barrister Muhammad Abul Kalam from UK regularly inspired me to complete this work, so thanks are also due to them.

Words fail me to express my appreciation to my wife Mrs. Nazneen Nahar Nazu, whose dedication and love inspired me lot to take this work at this age. I also express my heartfelt thanks to my two boys Nafi and Naiem for their sacrifice and love. Special thanks to my sister Fatema Khanom (Head master of a Govt Primary School), only uncle (Chacha) Md. Mokbul Hussain, father in- law Md. Abdul Matlib, mother in-law Fazlitaunnessa Khanom, for their encouragement and dua to complete this work in due time.

I am also grateful to my friend Md. Hafizur Rahaman Pir, Associate Professor of Botany, Md. Azizur Rahaman Laskar, Professor of Botany (Rtd), Md. Abdul Khaleque, Assistant professor of Botany, Cousin Rumel Khan, Nephew Mahfuz, Niece Manna, for their diverse help while working in Jaintapur, Sylhet. Thanks also to Md. Saiduzzaman Evan, friend of my elder son and Arafat Hussain of my brother's law farm who helped me lot to set up picture and tables.

I am deeply indebted to the staff members of Department of Botany, Md. Zahirul Islam (Technical officer), Md. Fazlu Miah (Administative officer), Md. Zafar Hossain (LD Asstnt), Md. Sah Alam (Senior store keeper) , Md. Yunus (Accountant, BAPTC &B) , MS. Rebeka Begum (Technical officer), MS. Hazera Khatun (Dept. Librarian) for their all possible cooperation during my study.

Last but not the least I am also grateful to Md. Mizanur Rahaman, Md. Ful Miah of Department of Botany, Haradhan Mazumdar and Md. Zakir Hussain of Pharmacy Faculty of University of Dhaka for their Lab assistance.



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List of Abbreviations

BARI	Bangladesh Agriculture Research Institute
cm	Centimeter
^0C	Degree Celcius
cv	Cultivar
2,4-D	2,4-Dichlorophenoxy acetic acid
e.g.	Exampli gratia/ for example
EDTA	Ethylene Diamine Tetra Acetic acid
et.al.	eti alli or others
etc.	et cetra, and rest
eV	Electron volt
FID	Flame Ionization Detector
Fig/s	Figure / Figures
g	Gram
g/l	Gram per liter
GA_3	Gibberelic acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric Chloride
hr	Hour
HRC	Horticultural Research Center
i.e.	Id est (That is)
IAA	Indole-3, Acetic Acid
IBA	Indole-3-Butyric Acid
inch	Inches
Kn	Kinetin
l	Liter
lb	Liber balance
mg	Milligram
mg/l	Milligram/liter
MIC	Minimum Inhibitory Concentration

ml	Milliliter
mins	Minutes
mm	Millimeter
µm	Micrometer
MS	Murashige & Skoog (1962) Medium
MS_0	MS basal Medium
MS_1	MS Medium supplemented with BAP 1mg/l
m/z	Mass charge ratio
$Na(OCl)_2$	Sodium Hypochloride
0.1N	Zero point one Normal
Na_2EDTA	Sodium salt of ferric Ethylene Diamine Tetra Acetic acid
NAA	α-Nepyhalene acetic acid
NaOH	Sodium hydroxide
No.	Numbers
pH	Negative logarithm of hydrogen ion concentration
Ph.D.	Doctor of Philosophy
sec	Seconds
S.D	Standard Deviation
sp	Species
sq	Square
STG	Shoot Tip Grafting
T-20	Twenty twenty
TEA	Tri-Ethanol Amine
TLC	Thin Layer Chromatography
Viz.	Videli = namely
wt	Weight
w/v	Weight per volume
%	Percentage

Abstract

A study was carried out at the orchard of Citrus Research Station, Jaintapur, Sylhet during the years 2009 -11 to investigate plant characteristics, yield potential, floral biology, pollination behavior, flower & fruit drop, flower bud development, fruit growth & development and mitotic chromosomes of root tips of *Citrus macroptera* Mont.Var. *annamensis*. It is a citrus fruit crop under the taxonomic family Rutaceae, its English name is Melanesian or Annam papeda and Bangla called Satkara. The different aspects of the biology of ten selected germplasm were studied. Medium sized evergreen tree, height of (13-14 yrs) grafted plants 2.08 -2.92 m, base girth 27- 55 cm, canopy shape was both spheroid and irregular and tree growth habit mostly erect, thorny, tree vigor both high and medium and canopy diameter was 2.03 - 3.66 m. The shoot tip color was pale green to light yellowish at very early stage, leaf lamina and apex shape were rounded and lamina margin crenate, lamina length 6.07 cm and width 4.66 cm, petiole slightly longer than leaf lamina and in some cases double than lamina, mean petiole length 8.02 cm, lamina and petiole ratio was 1: 1.31. Winged petiolate leaf which was longipetiolate, petiole shape obovate and lamina shape rounded.The Satkara flowers are small, bisexual, flowering start in February and end in March, but maximum flowering month is February, flowers arrangement both solitary and clustered, inflorescence cymes, initially bud color is purple but later cross purple margin on the top, open flower color is white but exceptionally it is purple or pale. A mature flower bud size was 12 -14 mm X 8 – 10 mm, pedicel length 1.0 -1.5 mm, calyx cup shaped and diameter 2.0 - 2.42 mm, number of sepals usually 5 but rarely 4, petals usually 5 but sometimes recorded 3 or 4, petals oval shaped and size 7.00 - 7.20 mm X 4.2 - 6.0 mm. The flower was protogynous and the stamens were longer than pistil. The pistil was small and stud shaped and length was 3.12 mm, style very short and fixed in the cavity of ovary and length ± 1 mm, Ovary size 1.16 mm X 2.33 mm, locules per ovary is 14, stamens free and numbers 24 - 29, stamen length 6.05 mm, stigma capitate and size1.41 mm X 1.68 mm, stamens longer than pistil and 30 - 40% rudimentary pistil were recorded. Pollen shape rounded and diameter 25.44 µm, viable pollens 75.08% and germination rate was 34.97%. The fruit shape was obloid but frequently spheroid, fruit base truncate, fruit apex rounded and depressed, rind color light yellow to green yellow and light green, albedo color white, smoothness of rind surface "smooth & pitted", bitterness of peels were nil, low, medium and high. The pulp color was creamy white and taste highly sour, juice color creamy white, seed shape semi deltoid, seed coat color creamy, harvesting month of fruit is October, weight 165 - 260 g per fruit, size 4.50 cm – 6 cm X 5.2 - 7.25 cm, rind thickness 8.2 mm, number of segments 14, juice content 131 ml per fruit, days required from fruit set to harvest 175.55, fruits per plant 12.2 and seeds per fruit was 6 -11. During 2009 the average flower drop was

92.81% and after 120 days the fruit set was 0.88%, whereas during 2011 flower drop was 98.32% and after 120 days the fruit set was 0.24%. In case of Satkara bud period was 32 - 35 days and maximum drop occurred before anthesis, stigma became receptive and dehiscence of anther was done before anthesis, many rudimentary or abortive pistils were present, self pollination occurred in Satkara. There are six stages of flower bud development, maximum flower buds dropped at early stage. The growth and development of fruit was divided into seven stages, from blooming to fruit maturity required 200 - 230 days and at harvesting stage the color of fruit skin became yellowish green, oil glands were visible in naked eye, growth became slower during June but from mid July to August rapid growth occurred and from mid August growth was reduced, mature fruit size was 4.70 × 6.47 cm. Mitotic chromosomes of Satkara were studied by Orcein staining method and CMA by Fluorescent method. All slides showed 2n = 18 without any numerical variations. Individual chromosome length ranged from 0.05 to 1.89 µm, total length of 2n complement was 24.30 µm, relative length of each chromosome ranging from 0.04 to 0.08. No satellite or secondary constriction was found. GC rich many CMA - banded dot like yellowish positive bands (10 - 15) shown in mitotic inter-phase and prophase, 8 CMA - positive bands in mitotic metaphase. Total length of CMA - positive banded region was 4.45 µm which covered 18.3% of the total chromatin length. **Phytochemical** study was conducted to identify, estimate and biological evaluation of the chemical and nutritional components, major acid contents of the fruits. The ash value of the peel 0.89%, moisture 75.53%, protein 1.02%, fat 0.85% and carbohydrates 18.98% were found. The pH value for peel 4.32, juice 2.45 and TSS for peel 24.47 and juice 12.43 were recorded. Crude fiber and energy of peel were 2.46% and 90.0 Kcal. Ascorbic acid (vit C) 5.58 mg and 53.07 mg, citric acid 4.53 mg and 55.05 mg, malic acid 1.43 mg and 4.54 mg per 100 g for peel and juice were recorded respectively. The antibacterial activity of the ethanolic peel extracts of Satkara was evaluated using disc diffusion method. Both Gram positive and Gram negative bacteria and fungi were used in this experiment. None of the Gram positive bacteria except *Staphylococcus aureus* showed any zone of inhibition. Whereas in case of Gram negative bacteria only *E.coli, Vibrio mimicus* and *Shigella boydii showed* 8 mm zone of inhibition. All the fungi showed 8 - 9 mm zone of inhibition. The overall results indicated the mild antimicrobial activity of the peel extracts.The ethanolic extract of the peels (ECM) was subjected to brine shrimp lethality assay.The lethal concentration LC_{50} value of ECM was found to be 2.53 µg/ml, which is compared to reference standard Vincristine Sulphate (VS) with LC_{50} of 0.45 µg/ml following regression analysis. The steam distillation of the 100 g fresh peels of the fruits afforded 120mg of oil (yield 0.12%). Analyses both by gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS) of the sample

resulted in the identification of 25 terpenoids, predominantly monoterpene hydrocarbons, accounting for 97% of the total oil, and limonene (73.5%) as the major component. While the oil did not exhibit any *in vitro* free-radical-scavenging (DPPH) activity, it showed considerable antibacterial activity against *Bacillus cereus, B. subtilis, Escherichia coli* and *Staphylococcus aureus* with the MIC values ranging from 1.25 to 5 µg/ml the antimicrobial activity of the extract and volatile oils is due to the presence of the terpinoids in the extract and volatile oil. The fresh peels extraction contained phytochemical constituents like tannins, saponins, flavonoids, steroids, terpinoids and cardiac glycoside but alkaloid was absent. **The explants for *in vitro* study** were collected from field plants (Sylhet) and pot grown plants in Botanical garden of University of Dhaka. Five sterilization procedures (P1 – P5) were followed for surface sterilization. Two types of media were used for surface sterilization, MS_0 (MS basal medium) and MS_1 (BAP 1 mg/l supplemented medium). No sprouting of shoots was recorded in explants cultured on MS_0 media but sprouting of shoots was recorded in MS_1 media. In case of P_1 (0.1% $HgCl_2$ solution for 10 minutes), P_2 [5% Na $(OCl)_2$ for 10 minutes], P_3 (0.1% $HgCl_2$ solution for 15 minutes) and P_4 [5% Na $(OCl)_2$ for 15 minutes] contamination rate were 100% for both field and pot explants in both media, but in P_5 [5% Na $(OCl)_2$ for 10 minutes + 0.1% $HgCl_2$ solution for 10 minutes] only 20% contamination was recorded for pot explants and no contamination for field explants in MS_0 medium, whereas in MS_1 medium 20% contamination was recorded for pot explants and 100% for field explants The sprouting of shoots were also higher in P_5(80%) than others, P_1(20%), P_2(40%), P_3(20%) and P_4(20 %). The sprouted explants collected from pots were contamination free whereas sprouted field explants were contaminated later. The pummelo seedling used as rootstock for *in vitro* micrografting and seeds were grown in MS basal medium under *in vitro* condition. After 20 days only 74% of the seeds were germinated but after 25 days further any germination was not recorded. The seedling length was 3.20 cm at 25 days and 4.27 cm at 30 days. 3 – 4 mm size buds were joined on 4-5 cm decapitated pummelo shoot. After 7 days of grafting only 20% buds were joined and 40% plants were contaminated after 10 days. The 50% of the failure buds were dropped after 10 days. Finally, all joined grafts were contaminated and within 20 - 25 days all bud along with stocks became dried. In case of *ex vitro* grafting, buds from *in vitro* grown were grafted with *ex vitro* grown pummelo seedlings. After 25 days, the 30% buds joined with stocks and 70% buds became dried and dropped. No new bud was joined after 25 days and remaining 70% were dried and dropped. Grafted plant length with single leaf became 4.2 cm at 35 days, 6.5 cm with two leaves at 45 days, 7 cm with three leaves at 50 days, 7 cm with four leaves at 55 days. The length became almost same (7.4 cm) after sixty days. For *in vitro* regeneration of shoots from nodal explants, three concentrations of BAP were used alone. Shoot inductions were started after 6 days of inoculation and maximum induction done within 10 days. The highest percentage of shoot

induction was (85.54%) recorded in 1 mg/l BAP followed by 1.5 mg/l BAP (40.25%) and 0.5 mg/l BAP (25.00%). The highest number of regenerated shoots and number and usable shoots were recorded highest in 1 mg/l BAP supplemented medium. Average length of microshoots was also highest (18.3 mm) in 1 mg/l BAP medium. Microshoots regenerated in BAP supplemented medium was subcultured on same medium after 5 weeks. The results were not satisfactory because some of the shoots were elongated vertically without any new branching, few were not elongated and medium became brown. Less than 10%.of the shoots were grown successfully with new shoots. The elongation of shoot on hormone free medium was good but shoots were weak and yellowish green. Microshoots of 16 -18mm long with 3 - 4 leaves were directly transferred to the sterilized moist pot soil. Initially the plants were grown under growth room condition, then after 4 days plantlets with poly bags transferred to hardening room. The survival rate of the microshoots was 80%.

Introduction

Introduction

Citrus is a common term and genus of flowering plants in the family Rutaceae. The Latin word citrus was borrowed from ancient Greek Kedros "cedar, juniper" (Kimball, 1999). Some believe this was because Hellenistic Jews used the fruits of *Citrus medica* during Sukkot (Feast of the Tabernacles) in place of a cedar cone, while others state it was due to similarities in the smell of citrus leaves and fruit with that of cedar (Spigel-roy, 1996). Citrus is considered as one of the most important fruits of the world and in terms of acreage, it occupies probably the third position among subtropical fruits (Randhawa & Srivastava, 1986). The fruits are liked by one and all, not only for their attractive appearance, penetrating flavor of the peel, but also for excellent quality as foods.

Citrus macroptera Mont. Var. *annamensis* known as Melanesian papeda or Annam papeda in English and in Bangla it is called Satkara. It is a minor and semi wild fruit crops and traditionally used in culinary and tasty pickle preparation. These fruits are well known in greater Sylhet division of Bangladesh and in India it is grown in the north eastern regions of Shella and Dowki near Cherrapunji in Meghalaya, the Jampui Hills of Tripura & Mizoram, and the Chendel District of Manipur (Das and Kumar, 2010).

So far known, there is no production statistics of Satkara available in Bangladesh. But apparently it has been shown that the availability and sale of this fruit in different markets of Sylhet (village to town) increasing day by day. About 216 metric tons of Satkara is being imported every year from India through different borders of greater Sylhet division (Appendix - 5). But major portion of the fruits come from the neighboring states of India through illegal ways (Anonymous, 2010).

Recent information from different sources suggested that productions of Satkara in our country is not satisfactory because of the following reasons, these are:

1) Lack of suitable varieties,
2) diseases among the existing varieties,
3) lack of proper management ,
4) lack of extensive and sustainable research works and
5) due to slow growth of seed born plants.

The information and research findings on Satkara plants are very limited. Bangladesh Agricultural research Institute (BARI), HRC division, Gazipur, Bangladesh mentioned in one of their publications only the effect of irrigation on Satkara plants (2008 - 2009). The BARI authority used the botanical name of Satkara is *Citrus macroptera* only, which is partial of the full name of this crop. The full name is *Citrus macroptera* Montrouz. Var. *annamensis* Tanaka. Because, the *C. macroptera* has another variety name *C. macroptera* Mont. Var. *kerrii* Swingle [Plate: **i** (e)] found in Northern and central Thailand, North Vietnam, Tonkin, Southern China and Southern Yunnan Province, and its common name is Kerr's Thailand papeda.

Because of diverse aspects of the present study, the whole research work is divided into three major parts, like **Biology, Phytochemistry,** and ***In vitro* propagation.** The introduction are presented under the following headings,

A) Biology
B) Phytochemistry
C) *In vitro* propagation
D) Origin and distribution
E) Soil and climate
F) Classification and
G) Economic importance of Satkara.

A) Biology

For the improvement of any crop, there need to have diversity of existing varieties with high yield along with other desirable traits. In case of Satkara, so far we know there has no information of high yielding varieties or any recommended varieties in our country, Bangladesh Agricultural Research Institute (BARI) trial the some germplasm of Satkara at Jaintapur, Sylhet and Akbarpur of Moulvibazar districts. The BARI scientists stated that 14 - 15 years back they collected some germplasm (scion) from different localities of Sylhet region and grafted on pummelo rootstock and other citrus species. So, apparently observed some variation among the rootstock grafted plants. Selection is one of the methods to obtain new improved varieties from the existing gene poll. Before starting selection or other breeding program, we need to characterize the selected germplasm.

Satkara have three main flushes of growth. The main one occurs in late winter or early spring (spring flush). Two additional ones occur at the end of the June drop (summer flush) and late in September (autumn flush). Only vegetative shoots are formed in the summer and autumn flushes of growth. Inflorescences are formed in the spring flush of growth as a response to the low winter temperatures.

Flowering is a critical step in fruit formation. No flowers mean any fruit, and when flowers number is low crop load may be limited. In most cases, however, citrus trees form a large number of flowers exceedingly higher than the final number of fruits harvested, which usually is a very low percentage of the initial flower numbers. Hybridization program is essential to obtain the high yielding variety with desire characters. The prerequisites for an effective hybridization program included the floral morphology, biology, anthesis, dehiscence, pollen viability, stigma receptivity etc. (Ahamad, 1996).

Fruit production is a reproductive process initiated at the time of flowering. The first stage of flower initiation is followed by flower growth, pollination and fertilization, growth of the fruit to maturity or harvest. The fruit production is highly influenced by the climatic factors. Without the effects of environmental and climatic factors, fruit production is functionally is a function of flowering and fruit setting.

Like other citrus species Satkara is also regarded as modified berry called hesperidium. In case of Satkara the peel is edible in culinary and pickle preparation. In case of immature fruit, the peel along with pulp used in curry or somehow for more sourness. The pulp juice is also used by many people as a drink but generally it is not used because of high sour taste. Starting from flower initiation Satkara takes a long time for fruit maturity, about 210 - 220 days to become full mature. Therefore, the requirements of the fruit for its proper development are not similar throughout the whole growing season. So, it is necessary to know the growth and development stages of the fruit for better production.

The amount and quality of pollen produced by a flower is an important component of fitness. Pollen quality is often equated to pollen viability, i.e., the production of pollen grains that are viable. But all viable pollens may not be germinated, so it is important to know the germination percentage of pollens. The pollen viability estimate can be used as an indication of possible interference from genetic (Eady *et al.,* 1994. Twell, 1995). The pollen fertility is variable among the citrus species (Agarwal, 1993). Although various stains have been used to studies of pollen viability, staining may not indicate true viability. Erlanson, (1931) reported that many apparently perfect grains were unable to affect fertilization. On the other hand, Visser *et al.,* (1977) called stainable pollen grains "normal pollen" but considered them as having only the potential to germinate.

The genus *Citrus* has large number of species and varieties under the family Rutaceae. Within this genus, high cross compatibility has led to natural interspecific and intergeneric hybrids resulting in numerous morphological variations. This variation has been transmitted to progenies by nucellar embryoney. Because of their heterozygosity, the taxonomic systems among citrus are still controversial (Tanaka, 1977).

The discrimination of citrus chromosome at a somatic metaphase is difficult because of their small size (1.5 µm to 3.5 µm) and similar morphology. Chromosome at pro-metaphase may be more suitable for karyotyping in citrus (Befu *et al.*, 2000). The cytological characterization of citrus species could help in the identification of a particular genomic variant or for the detection of the true hybrids in breeding

programs as well as studies of karyotype evolution of the groups (Guerra *et al.*,1997).

B) Phytochemistry

The Satkara fruits are used as a traditional medicine by the local tribes of Assam in India (Ghosh,1990), Chowdhury *et al.,* (2008) have recently reported the antioxidant activity of the crude extract of this plant. While there are no reports available to date on any phytochemical studies on this particular variety of *C. macroptera*, a few previous studies on other variety of *C. macroptera* revealed the presence of a quinolone alkaloid and coumarins.

Along with vitamin C, citrus has contained notable essential nutrients, like glycaemic and non-glycaemic carbohydrate (sugars and fiber), potassium, folate, calcium, thiamin, niacin, vitamin B_6, phosphorus, magnesium, copper, riboflavin, pantothenic acid and a variety of phytochemicals. Being a plant food it contains no cholesterol, fat or sodium. Citrus fruits are important among populations who need to overcome and prevent micronutrient deficiencies as well as those concerned with problems of over nutrition, obesity and diet related chronic diseases (Economos,1998).

In recent years, the general public has shown an increased interest in the use of herbal medicines in preference to synthetic drugs. This is based on the belief that natural products are intrinsically less dangerous and can be obtained at a lower cost. However, most natural products contain a complex mixture of components of which only one or a few are active. Identification of the active component can lead to the synthesis of more potent analogues that can be readily formulated into more traditional dosage forms. Volatile oils, also known as essential oils are lipophilic compounds containing volatile aroma compounds. They are generally isolated from non woody plant materials by distillation methods, solvent extraction or cold expression and used in perfumes, cosmetics, flavoring food and drinks and for scenting. Medicinal uses proposed by sellers of essential oils vary from skin treatments to cancer treatment. These are often based on historical uses of these oils. These claims are now subject to regulations in most countries and there is a need to ascertain some of these traditional uses scientifically (Lwu *et al.,*1999).

Tangerine oil is obtained from the fruit peel of *Citrus reticulate* Blanco family Rutaceae. It is traditionally used as an antiseptic, antispasmodic, stomachic, sedative, diuretic and to improve circulation (Burkill, 1984 and Odugbemi, 2006). The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity. Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile oils have regained momentum. Increase in the emergence of new bacterial strains that are multi-resistant coupled with the non availability and the high cost of new generation antibiotics have resulted in increase morbidity and mortality (Aibinu *et al.,* 2003. Amit and Shailendra, 2006). Hence need to look for potent antimicrobials from other sources.

The limnoids present in citrus fruits such as grape fruits have been shown to inhibit the growth of cancer cells in cancers of the colon, mouth, lung, stomach and the breast across animal and cell studies (Mandadi *et al.,* 2007).

The citrus fruits have long been valued as part of a nutritious and tasty diet. The flavors of citrus are used in perfumes, foods, drinks, culinary and other purposes. It is well established that citrus and citrus products are a rich source of vitamins, minerals and dietary fiber (nonstarch polysaccharides) that are essential for normal growth and development and overall nutritional well-being. The essential oil extracted from *Citrus macroptera* Mont. Var. *annamensis* (Satkara) found well flavors and there has good probability to use in different commercial purpose.

C) *In vitro* propagation

Like other citrus plants (lemon, lime, etc.) the Satkara plants do not grow easily, here needs good rootstock for grafting. In case of Satkara, the BARI authority used pummelo (*C. grandis*) and other local citrus rootstock. According to BARI scientists the growth of grafted plants are very slow and also very weak. Vegetative propagation should be employed in citrus to ensure uniform quality and regular bearing (Mankand, 1994). Various citrus species are propagated by different methods, but the most common method of Satkara is seeds or grafting.

In nature, the methods of plant propagation may be either asexual or sexual. Sexually propagated plants demonstrate a high amount of heterozygosity since their seed progeny are not true-to-type unless they are derived from inbred lines. Asexual production on the other hand, gives rise to plants that are genetically identical to the parent plant and thus permits perpetuation of the unique characters of the cultivars.

Strategies for application of plant tissue culture tools and techniques would include clonal propagation which implies large scale *in vitro* propagation of plants of uniform quality, produced more rapidly and abundantly than possible by conventional methods of classical breeding. This branch also has alternatives for crop improvement through genetic manipulation, conservation of plant genetic resources and production of valuable chemicals. In recent times, it is an essential part of biotechnology research in different institutes, universities and companies to purpose the development to improve plants for agriculture, horticulture and forestry (Carlson 1977, Amato 1978, Scowcroft and Larkin, 1982 and Skirivin, 1978).

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation. Clonal propagation through *in vitro* cultures in generally called micro propagation and it can be defined as "regeneration of plants from organs, tissues, cells or protoplasts" (Beversdrof, 1990) or the true-to-type propagation of selected genotype using *in vitro* culture techniques (Debergh and Read , 1990).

Micropropagation technology provide many advantages over conventional propagation methods such as product development in speedy manner, informing in product development, large population can be produced in small growing place, quick multiplication of elite and genetically engineered products, *in vitro* storages of germplasm and production of materials throughout the year.

One of the familiar methods of *in vitro* propagation is the regeneration or sprouting of plantlets from nodal explants of citrus plants. 10 – 12 mm long and 5 - 6 weeks old stem part with single bud cultured on MS media supplemented with different concentration of growth regulators. The success rate is also satisfactory (Bonzid, 1975 in sweet and sour orange. Sim *et al.,* 1989 in *Citrus mitis*. Amin & Aktar, 1993 in pummelo. Begum *et al.,* 2001 in pummelo). But explants collected from field grown plants and their regeneration is not easy because of high rates of

contaminations (Altaf, 2006: Samarina *et al.,* 2010). Several workers tried and became successful, but rate was not satisfactory (Marin & Duran, 1991 in *Citrus sinensis*. Altaf, 2006 in Kinnow. Miah *et al.,* 2008 in *C. macroptera*. Sen & Dewan, 2010 in Citronelo).

Grosser, (1994) mentioned that citrus are generally propagated through bud grafting, cutting or layering. Propagation is therefore, limited to the period when buds are available. These conventional techniques are also not free from risk of perpetuating inborn pathogens. However, *in vitro* micrografting/propagation techniques can overcome the some constraints to citrus improvement.

A method to recover citrus plants free of all virus and virus like diseases and without juvenile characters are really needed. The first attempt in this direction was made by *in vitro* shoot tip culture, a technique extensively used to recover healthy herbaceous plants. Murashige *et al.,* (1972) were able to obtain a few citrus plants by grafting shoot tips from diseased plants on young rootstock seedlings grown *in vitro*. Some of these plants were free of exocortis virus and did not have juvenile characters. Since then, several aspects of the shoot tip grafting (STG) technique have been published (Navarro *et al.,* 1975, 1976, 1980).

Presently shoot tip grafting in *in vitro* has been extensively used to recover the disease free plants from the commercial citrus varieties and become a potential tool for separating virus and virus like agents (Navarro, 1981). This technique is based on the fact that the apical meristems contain no or little virus tire and meristematic cell grows faster than all viruses. Therefore, the production of disease free foundation plants by micrografting remaining the only mean to supply disease free bud sticks to the growers (DeLang, 1978). Successful shot tip grafting and disease free plants were obtained in different citrus species by several workers (Juarej *et al.*, 1990 in Citron. Sharma *et al.,* 2007 in Kinnow. Fifaei *et al.,* 2007 in naval orange).

D) Origin and Distribution

Citrus is a collective general name, embracing a number of species and varieties of fruits, known all over the world. Although, the cultivation of various citrus fruits has been known to mankind since a hoary past, the actual country of origin of the genus has been a controversial subject. Citrus fruits had been grown for many centuries in China and reached a considerable advanced stage of cultivation before they became known in other parts of the world.

It is believed that most of the species under the genus citrus are native to tropical and subtropical regions of southeast Asia particularly, India, China and in the region between these two countries, the northeastern region of India is considered as one of the natural homes of citrus – at least for a few species.

The original home of the vast majority of citrus fruits and their relatives is considered to be the warm southern slopes of the Himalayas in northeastern India and adjacent Myanmar (Burma), Southern China and Indo-China. Thus, some species must be native to China, some to India and others to the regions between these two countries and Malaya Archipelago. According to Bhattacharya and Dutta, (1958) the two species of Eucitrus, *Citrus indica* and *Citrus assamensis* and three species of Papeda, *Citrus ichangensis*, *Citrus iatipes* and *Citrus macroptera* are indigenous to Assam. They opined that *Citrus aurantium*, *Citrus magaloxycarpa* and one variety of *Citrus reticulata* were also indigenous to Assam. From there, these were later introduced and cultivated in other parts of the world.

Burke, (1967) has observed that many citrus fruits originated in Assam in the north eastern part of India and these include the citrus species like *ichangensis, indica, latipes, macroptera, jambhiri* (rough lemon), *limonia* (Rangpur lime), *assamensis, karna, limettioides* (Indian sweet lime), *aurantium* (sour orange) and progenitors of the citron, lemon and some mandarins.

Dutta, (1958) while discussing the origin and history of citrus fruits of Assam, reported that the *C. indica* Tan., *C. latipses* Tan., *C. macroptera* Mont., *C. medica*

L., *C. inchangensis* Swing., *C. aurantium* Linn, *C. sinensis* Osbeck, *C. jambhiri* Lush. and *C. assamensis* are undoubtedly indigenous to Assam.

Hodgson *et al.,* (1967) felt that species standing was justified in the cases of Rangpur lime, Rough lemon, Sweet lime, Galgal, Kharna Khatta, Kichili, Gajanimma, Indian wild orange and Khasi papeda, but were doubtful of the justification of granting species rank to Adajamir of Assam, sadaphal and Satkara. They opined it would be justified if Adajamir of Assam is treated as botanical variety of Gajanimma of South India- *C. pennivesiculata* var. *assamensis* and Satkara as botanical variety of *C. macroptera* - *C. macropetera* var. *annamensis* .

Citrus is an economically important fruit crop, ranking almost as high as apples in world production and trade. The phylogeny and taxonomy of citrus fruit are complex, confusing and controversial due to the genetic heterogenecity of the genus, as well as its polyembryonic nature and long generation time needed to carry out selection and recombination (Swingle, 1948. Nicolosi *et al.,* 2005). According to Carpenter and Reece, (1969) *Citrus macroptera* (wild orange) is a tree which grows in Indo-Chlna, Myanmar, Thailand, Indonesia, Malayasia and Papua New Guinea.

E) Soil and climate

Citrus macroptera grows well in deep, loose and well aerated soil. Ideal pH for citrus is considered to be 5.5 to 7.5 but evidence shows that it can be grown well in well drained highly acidic soil with sufficient moisture throughout the year.

Generally citrus fruit belongs to the tender evergreen subtropical to semi tropical climate. They can tolerate low temperature and are sun loving and sensitive to shading. The plant cannot survive under prolonged drought. The location and climate of Citrus Research Station, Jaintapur, Sylhet is following,

Land area	: 118.64 acres
Location Quarter.	: About 44 km north-east from Sylhet Divisional Head

Altitude	: 20 m
Latitude	: $25^0 8'$ N
Longitude	: $92^0 8'$ E
Total annual rainfall	: 6000 mm (approx.) per year
Temperature	: Min. 06^0 C (January), Max. 36^0 C (April)
AEZ	: 22/29
Soil Texture	: Sandy clay loam
Soil pH	: 5.2 - 5.6

F) Classification

Taxonomists have widely divergent views on the classification, and there does not seem to be a general agreement on the concept of genus and species. Studies on classification of Citrus have been undertaken by many workers in several countries. Significant contributions have been made in United States of America and Japan in this direction. Citrus can right be regarded as a universal fruit as the production in over 100 countries in all six continents. Furthermore it is the most important tree fruit crop in the world with current production for that of all deciduous tree fruits (apples, pears, peaches, plums etc.)

Any botanical classification of citrus fruit faces several difficulties. All citrus types are hybrids easily and new hybrids are continuously obtaining desired qualities such as seedless, juiciness and fresh taste. New hybrids spontaneously arise also by cross-pollination. The differences between a species, a new botanical variant and different cultivated (horticultural) variety has small differences in most cases. In the case of older varieties and hybrids the most modern methods of molecular research are sometimes needed to distinguish different citrus types from each other.

There is a good deal of concepts in the views of classification of citrus, primarily due to divergent concepts about the constitution of a valid species. Up to this time, there is no classification, which has received general acceptance by pomologists and botanists. Although more than a dozen of scholars, notably in USA and Japan have attempted to classify citrus, only the words of W. T. Swingle (USA) and T. Tanaka

(Japan) are noteworthy. Classification of Swingle and Tanaka are two extremes. Swingle, (1948) recognized only 16 species whereas Tanaka, (1954) described as many as 144 species, Swingle also divided genus citrus into two subgenera, viz., Eucitrus having 10 species and Papeda having 6 species. In India Bhattacharya and Dutta, (1956) have also classified the citrus fruits of Assam. According to Swingle, (1948) the species C. *macroptera* (Mont.) is under the subgenus papeda.

It is thus clear that many attempts have been made for classification and grouping of citrus fruits, but two systems of citrus taxonomy and nomenclature by Swingle and Tanaka have found wider adaptability. Reece, (1967) considers that Swingle's system comes closest to the idiom of citrus taxonomy and many authorities do not accept the Tanaka's system with its excessive splitting of citrus forms into species. Hodgson, (1967) Singh, (1967) and Singh and Nath, (1979) have however suggested some compromise between the two systems. Presently, Swingle system of classification is widely prevalent. His system of classification seems to be sufficiently comprehensive and elastic for general application with certain minor adjustments. It can be made to accommodate most of the known Indian citrus varieties.

Nowadays, only the International Botanical Congress, which has been held every five years since 1900, can change the name of a plant according to rules set out in the International Code of Botanical Nomenclature. The currently valid code is the Vienna Code of 2006 available on the Internet. Records of all botanical conferences since 1950 are kept at the International Association for Plant Taxonomy in Vienna, headed by D.J. Mabberley, President since 2005.

According to Swingle (1948) the basic classification of genus citrus up to species is as follows:

Genus : Citrus (2 sub genera, 16 species, 8 botanical varieties)

Sub genus-1 : Eucitrus (10 species)

1. *Citrus medica* (Citron)
2. *C. limon* (Lemon)

3. *C. aurantifolia* (Lime)
4. *C. aurantium* (Sour orange)
5. *C. sinensis* (Sweet orange)
6. *C. reticulata* (Mandarin)
7. *C. grandis* (Pommelo)
8. *C. paradisi* (Grape fruit)
9. *C. indica* (Indian wild orange) and
10. *C. tachibana* (Tachibana orange).

Sub genus - 2 : Papeda (6 species)

1. *Citrus ichangensis* : (Ichang papeda).
2. *C. latipes* : (Khasi papeda).
3. *C. micrantha* : (Small-fruited papeda).
4. *C. celebica* : (Celebes papeda).
5. *C. macroptera* : (Anam papeda & Kerr's Thailand papeda).
6. *C. hystrix* : (Mauritius papeda, Kaffir lime).

For better understanding and to avoid some confusion, the description of six papeda species are presented below:

1. ***Citrus ichangensis*** Swingle.

Common name : Ichang papeda.

Distribution : West-central and southwestern China: Hupeh, Szechwan, Kweichow, and Yunnan province.

A spiny shrub or small tree usually 5 - 15 ft. [1.5-4.6 m] high; leaves narrow, 4 - 6 times longer than wide, mostly 8 - 11.5 X 1.8 - 3 cm, petioles very large, broadly winged, obovate. Flowers 2.5 - 3 cm diameter, 5 merous; white; stamens 20, style very short, stigma nearly as large as the ovary; ovary with 7 - 9 locules, fruits small, glabrous, 3 - 4 cm diameter [Plate: i (a)]

2. ***Citrus latipes*** Swingle.

Common name: Khasi papeda.

Destribution : Northeastern India: Khasi Hills; northern Burma;

A thorny tree similar to *C. ichangensis* but having leaf blades more variable in size and shape. The flowers are borne in small axillary racemes with 5 - 7 flowers. Flowers are much smaller and 4 merous. However, the fruits are borne singly and resemble those of *C. ichangensis* except for having a thicker peel [Plate: i (b)]

3. ***Citrus micrantha*** var. ***microcarpa*** Wester,

Common name : Small-fruited papeda. *Native name*: Samuyao.

Destribution : Southern Philipines: Cebu and Bohol.

A shrubby tree, 4.5 meters tall, with slender branches and small, weak spines; leaves broadly winged, flowers in compact axillary or terminal cymes, 2 to 7, small, white, with trace of purple on the outside; stamens 15 to 18, ovary very small, globose to obovate; locules 7 to 9 [Plate: i (c)]

4. ***Citrus celebica*** Koord.

Common name : Celebes papeda.

Distribution : Northeastern Celebes, Southern Philippines.

Leaves are small, lanceolate and winged petioles larger than the blades. Flowers occurring in 3 to many flowered spikes or small cymoid clusters in the axils of the leaves, flowers medium-sized, 4-5-merous,ovary reversed pyriform,17 - 20 locules, style short, 5 - 6 mm; stamens short, free; fruits large (10 cm diam.), globose, with a very thick peel (about 3 cm) [Plate: i (d)]

5. ***Citrus macroptera*** Montrouz.

This species was discovered by Father Montrouzier on the Island of Art, situated a few miles to the northwest of the north end of New Caledonia. This species is characterized by large leaves, sometimes 10 or 12 cm long (including the very large winged petiole) and 5 cm wide, it has sub globose fruits the size and shape of an orange, but with very little juice.

Citrus macroptera has two sub species/varieties and one hybrid species. These are the followings:

5(a) *Citrus macroptera* var. ***kerrii*** Swingle

Common name. : Kerr's Thailand papeda.

Distribution. : Northern and central Thailand; North Vietnam: Tonkin; southern China: southern Yunnan Province.

The leaf blades are sometimes ovate, rounded at both ends, petioles broadly winged, obovate, often slightly larger than the leaf blade. Larger fruits, 8 - 9 cm diameter with 12 - 13 segments, peel 12 -14 mm thick. The spines on vigorous, young, vertically-growing shoots are very long (6 - 7 cm), straight, sharp, arising nearly at right angles to the direction of the twig, but on fruiting branches they are usually only 5 -10 mm long or wanting altogether [Plate: **i** (e)]

5 (b) *Citrus macroptera* Mont. var. ***annamensis*** Tanaka.

Common name : Melanesian papeda. Local name, Satkara.

Distribution : Known only from the type locality. North-eastern India, Sylhet regions of Bangladesh.

Leaves very large, leaf blades ovate, petioles broadly winged; petiole wings obobate, truncate, a little longer than the leaf blade. Fruits medium sized, obovoid or spheroid, peel glabrous. Pulp vesicles scanty and very short. The fruit is 4.5 cm high and 5.2 cm wide, peel 3 - 4 mm thick [Plate: **i** (f)]

5(c) Hybrid of *Citrus macroptera*

Name : Moli kurikuri hybrid.

Locality: Fiji.

A hybrid of *C. macroptera*, very probably with the pummelo (*C. grandis*), was named *C. vitiensis* by Tanaka.

6. *Citrus hystrix* DC

Common name : Mauritius papeda, Kaffir lime.

Distribution : East Indian Archipelago, Ceylon, Burma, Malay Peninsula, Philippines; widely naturalized, native habitat uncertain.

Kaffir lime is the strong spice used in Indonesian and Thai cooking. Low tree or shrub,2 – 12 m high, densely branched and branching starts from the base, leaves 3 -15 cm long and 2.5 - 6cm wide. Petiole winged and almost same length of leaf blade. Flowers small, shortly stalked, 4 – 5 merous. Fruit pendulous globose, glabrous, with a tuberculate folded surface. Many scattered glandular oil dots in the fruit [Plate: **i** (g)]

The detail classification of Satkara plant has presented below,

Kingdom : Plantae
Subkingdom : Viridaeplantae
Phylum : Tracheophyta
Subphylum : Euphyllophytina
Class : Magnoliopsida
Subclass : Rosidae
Super order : Rutanae
Order : Rutales
Suborder : Rutineae
Family : Rutaceae
Subfamily : Citroideae
Tribe : Citreae
Genus : *Citrus*
Species : *macroptera*
Variety : *annamensis*
Botanical name: - *Citrus macroptera* Montrouz. Var. *annamensis* Tanaka
(Ref: **Genus:** *Citrus*
Family: Rutaceae
Nomen number: 314288
Place of publication: Bull. Mus. Natl. Hist. Nat. sér. 2, 2:164. 1930
Name verified on: 20-Dec-1988 by ARS Systematic Botanists. **Last updated:** 29-Jun-2009.)

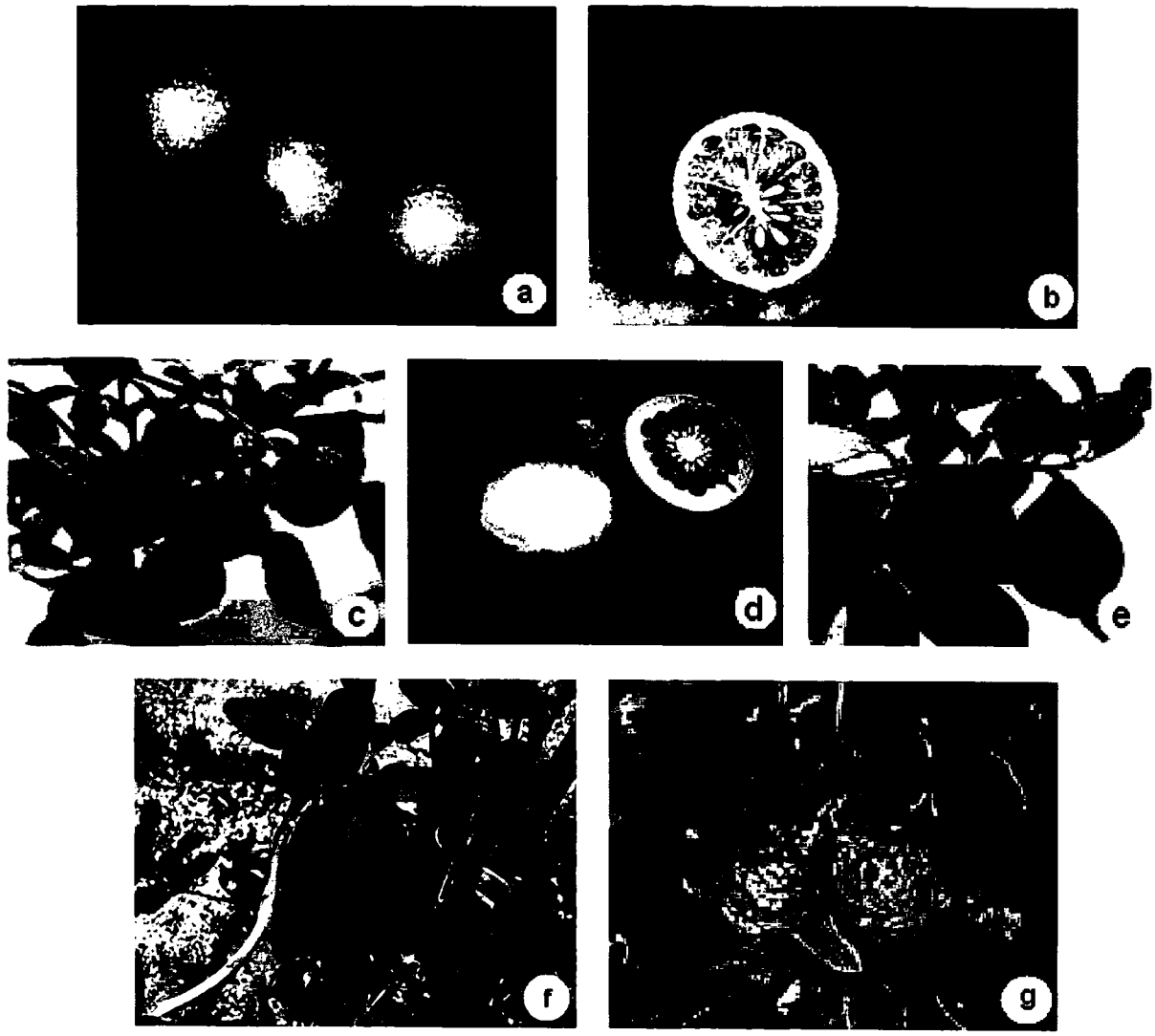

Plate: (i) Different species of papeda group a) *Citrus ichangensis* Swingle b) *Citrus latipes* Swingle, c) *Citrus micrantha* var. *microcarpa* Wester d) *Citrus celebica* Koord e) *Citrus macroptera* var. *kerrii* Swingle f) *Citrus macroptera* Montrouz. var. *annamensis* Tanaka, g) *Citrus hystrix* DC.

F) Economic importance

Citrus *macroptera* (Satkara) fruits are well known in greater Sylhet district of Bangladesh. In India, Satkara is found in the northeastern regions of India. According to Ghosh, (1990) Satkara is a semi wild species and used by local tribes of Assam of India for medicinal purpose and in cooking. In Bangladesh, both green and mature (yellow) fruits are used in cooking for flavoring curry, pulses and vegetables and also for pickle preparation. Fruits are also dried for preservation and used in culinary during off seasons. During season (July to November) per piece of standard sized fruit sell 15-20 Taka, but off season price vary from 70 -100 Tk.

Due to its good taste, nutrient value and high price (depending on size and season in local market) Satkara is economically important both in local and foreign market. Every year our country has earned a handsome amount of foreign currency by exporting this fruit in UK, USA, European and Middle-East countries where ethnic (Sylheti) people live extensively. In European market the slice of fresh fruit under packaging also available [Plate (**ii**) b)].

Generally the juice is not used directly like other citrus fruits due to their high acidic taste but juice is rich of Vit-C, citric acids and valuable micronutrients. From the ethno botanical knowledge of the local users suggested that the fruit has a good digesting ability. The Satkara pickle reduce flatulence and increase appetite, enhances digestive power and makes food delicious (Anonymous, 2011). The sweet flavor of essential oil of peel is used in perfume industries.

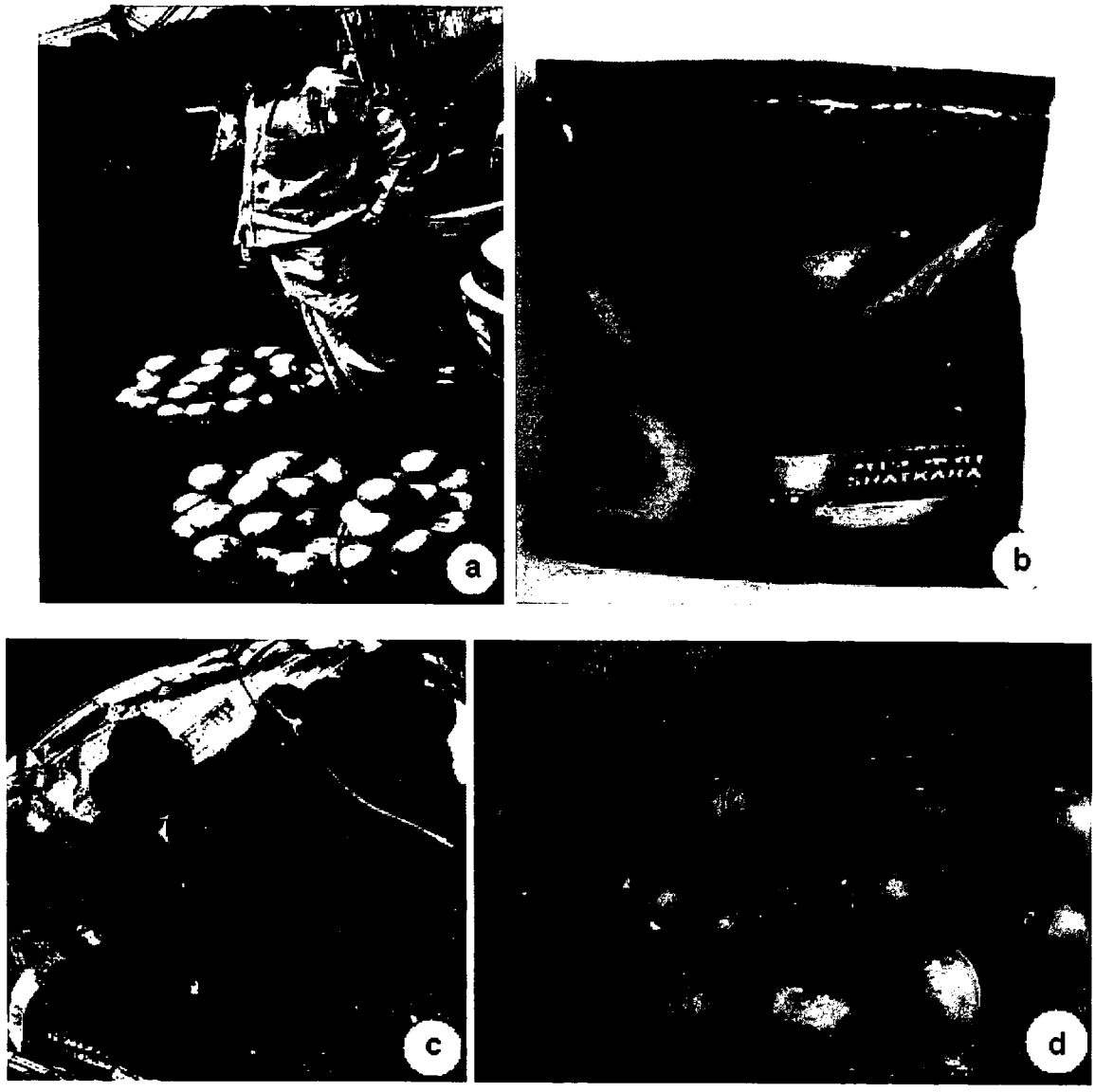

Plate: (ii) Vendors of Satkara; a) Ripen fruit selling in Sylhet town b) Package of the slices of fruit in European market, Brand : Crown Farms Item Code : 1480. Wt. 400g; Price: £1.4; c &d) Green and immature fruits selling in Sylhet town.

Review of Literature

Review of Literature

Citrus macroptera Mont. Var. *annamensis* is locally called Satkara because 'sat' refers to multiples of seven and the fruit generally contains fourteen segments. *C. macroptera* is so-named because of the large "wings" (ptera) on the petiole, which is as large as the blade of the leaf.

It is under the taxonomic family Rutaceae and one of the largest genus *Citrus* and Papeda group. This fruit is a minor and semi wild fruit crops and traditionally used in culinary, tasty pickle preparation, drinks, and perfume industry and also in medicinal purposes. The juices are generally not used like lemon, lime, orange and mandarin as raw because of high sour and somehow due to bitter taste, but juice contained vitamin C, citric acids and other mineral components. Satkara is well known and grow well in greater Sylhet division of Bangladesh and the people of this locality like it very much as a part of their culinary culture.

During the present work the studies are divided into three major parts and accordingly review of literatures are presented part wise.

Part – I : Biology,
Part – II : Phytochemistry and
Part – III : *In vitro* propagation.

In case of Satkara, the research findings and literatures are very limited, but in other citrus species the works are mostly available. From the various journals, books and internet searching the related references were collected and presented below;

Part – I : Biology

Bhattacharya and Dutta, (1956) descrived the two species of Eucitrus, *Citrus indica* and *Citrus assamensis* and three species of Papeda, *Citrus ichangensis*, *Citrus latipes* and *Citrus macroptera* are indigenous to Assam. They opined that *Citrus aurantium, Citrus magaloxycarpa* and one variety of *Citrus reticulata* are also indigenous to Assam. From there, they were later introduced and cultivated in other parts of the world.

Singh and Dhuria, (1960) described the details of the time of flowering and bud development in *Citrus limettoides*. The ratio of hermaphrodite flowers to male flowers varied from year to year and also during the season, averaging 8 or 9. Flower opening was greatest between 10 a.m. and noon, being slightly later on the shady side of the tree. Maximum pollen shedding occurred between 10 a.m. and 2 p.m., fertile grains being on average one third larger in diameter than sterile grains. Germination percentage of pollen, which averaged 51.24% fell as the season advanced. Stigma receptivity lasted 6 days and was at a maximum at the time of flower opening, but fruit set could be obtained 1 to 3 days before this. Numbers of fruit set was not obtained by hand selfing, nor was there any tendency towards parthenocarpy. Inflorescences with leaves attached produced more fruit than those without leaves.

Soost, (1964) reported that the Sukega grapefruit and hybrids of grapefruit have all failed to set fruits when self pollinated although their pollens of other varieties germinated *in vitro*. Frost and Soost, (1968) reported that mandarins are self sterile but cross fertile. They also reported the great differences in seediness of some pummelo varieties grown in different localities. Boyle, (1964) suggested that cross-pollination was the cause for the increased seediness in pummelos. Later work definitely demonstrates the presence of self-incompatibility in at least several pummelo varieties (Nauriyal, 1972. Aala, 1953. Soost,1964).

Hodgson, (1967) mentioned that this surface of the some young citrus fruits exhibit pubescence which is often retained till maturity. The surface of the fruit is generally pale yellow when ripe. Hossain, (1983) described that the skin color of pummelo as greenish yellow, light and yellowish with rough, medium rough and very rough surface.

Reuter *et al.,* (1967) descrived the description of *Citrus macroptera* var. *kerrii* Swing which is called Kerr's thailand papeda. The type specimens of this variety were collected in Nakawn Sawan Province in Thailand by A. G. F. Kerr, who stated that it is a "common tree in evergreen forest, growing up to 10 m high." Leaf blades are ovate, 3 - 7× 3 - 3.5 cm, petiole broadly winged and obovate and larger than blade (5 - 7 × 3 - 3.8 cm). Flower buds subglobuse, pedicels 3 – 5 mm long, calyx very small and saucer shaped, 1 mm long and 2 mm wide, petals 3.5 - 4.5 × 3 - 4 mm, stamens 16 - 20 and filaments are free. The fruits are subglobuse, 8 – 9 cm diameter , thick peel (1 – 2 cm) green rind 1 mm thick contains oil glands, albedo is white spongy tissue, the fruits show 12 - 13 segments. The pulp vesicles are numerous, 1 – 3 mm long with slender stalk.

Frost and Soost, (1968) proved that the thick, sticky liquid secreted from the stigma of mandarin serves not only to catch and hold the pollen grains but also provides suitable condition for their germination. Bud pollination has demonstrated successful pollen tube growth as much as four days prior to anthesis. The pollen grain absorbs moisture from the stigmatic liquid and forms the pollen tube. Pollen tubes grow through the style to locules of the ovary.

Frost and Soost, (1968) also described the stages of reproduction in citrus lemon. They divided the stages from bud formation to fruit set into eight stages viz. (A) bud probably having pollen mother cells before prophase of first meiotic division, (B) bud during meiosis in anthers, (C) bud at stage of young pollen grains, (D) bud nearly full grown, (E) flowers at time of pollination, (F) pistil just after fall of petals and stamens (about six days after pollination), (G) ovary just after abscission of style (about 10 of 15 days after anthesis) and (H) enlarged ovary after fruit set.

Guardiola *et al.*, (1982) investigated the effects of Gibberellic acid (GA_3) on flowering behavior of some citrus species. The application of GA_3 at any time from early November until bud sprouting, resulted in a significant inhibition of flowering in the

sweet orange [*C. sinensis* (L.) Osbeck] and the Satsuma (*C. unshiu* Marc.) and Clementine (*C. reticulata* Blanco) mandarins. Two response peaks were evident: the first occurred when the application was time to the translocation of an unknown flowering signal from the leaves to the buds. The second occurred during bud sprouting, at the time the flower primordia were differentiating. From the pattern of flowering, it appears that the mechanism of inhibition was similar irrespective of the timing of GA_3 application. There was an initial reduction in bud sprouting affecting selectively those buds originating leafless inflorescences. An additional inhibition resulted in a reduction in the number of leafy inflorescences with an increase in the number of vegetative shoots, suggesting the reversion of a floral to a vegetative apex. The inhibited buds sprouted readily *in vitro* but invariably vegetative shoots were formed.

Hossain, (1983) described that pummelo plants are closed or open headed and occasionally umbrella shaped. The main branches come out from the trunk at 0.76 - 1.22 m above the ground. The trunk is cylindrical in form and green when very young but the bark color turns grey to light brown as it grows older.

Hossain, (1983) stated that the flowers of pummelo are large, white; borne singly or in clusters having 4 - 5 sepals, 4 - 5 petals and 18 - 25 stamens. The stamens are united, ovaries globose and green. Faruque, (2001) reported that flowers of pummelo are white of light purplish in short cymes. Singh, (1995) reported that pummelo flowers are large, borne single in clusters, petals cream colored and stamen is ranged from 20 - 25 in numbers.

Hossain, (1983) reported that flowering of pummelo in Bangladesh started from first week of February and continued up to last week of March. This variation in flowering time may be attributed climatic conditions that prevail during different years.

Gonzales and Borroto, (1987) with the aim to increase citrus flowering under tropical conditions, two formulations of 2 - chloroethyl phosphonic acid (Ethrel and Flordimex), Alar (succinic acid - 2, 2 - dimethyl hydrazide) and Cycocel (2 – chloroethyl - trimethyl ammonium chloride) were applied during the flower induction period (December) to young "Valencia" orange trees at concentrations of 1000, 2500 and 5000 mg/l. These treatments effectively increased flowering, mainly at lower

concentrations. Treatment using Flordimex at 500 and 1000 mg/l concentrations resulted in flowering increase.

Lord and Eckard, (1987) studied the initial stages of inflorescence and vegetative development of *Citrus sinensis* L.(Washington Navel Orange). Bud break occurred in early December, floral initiation in early January, and anthesis in late March. To ascertain when a resting bud was determined as an inflorescence, they treated buds at four stages in development, ranging from resting buds to those producing floral organs, with gibberellins (GA_3) *in situ.* Examination of the shoots at maturity established that GA_3 caused complete reversion for buds in early stages of breaking and initiating floral organs, and no significant reversion once the sepals were present. The terminal flower may show reversion to the vegetative state even after a sepal has been initiated, but the lateral buds were determined at initiation.

Purseglove, (1987) reported that height of pummelo plants ranged from 5 m to 15 m. In Bangladesh, the plants attained a height of 5 -15 m (Hossain, 1983). Mondal and Amin, (1990) reported that most of the citrus plants are shrubs but few, such as pummelo become tree. Pummelo trees are large and spineless, whereas Rashid *et al.,* (1987) stated that pummelo trees are small.

Purseglove , (1987) mentioned that pummelo (2n = 18) flowers are large, borne singly or in clusters, 3 - 7 cm in diameter; calyx persistent, cup shaped, petals cream colored, stamens 20 - 40 in groups, filaments white and those of each, group partially united at base, anthers 4 celled, yellow in color and ovary with 11 - 16 locules. Most citrus are self and cross compatible. Stamens and stigma mature at the same time, the stigma is receptive for 6 - 8 days, the flowers are entomophilous and are visited mainly by bees. In artificial pollination anthers are removed in bud and the flowers are bagged.

Garcia-Luis *et al., (*1992) investigated the effects of low temperature on bud dormancy and flower induction of Owari Satsuma mandarin (*Citrus unshiu* Marc). They used potted trees and *in vitro* bud cultures. In potted trees the chilling treatments released bud dormancy and enhanced both sprouting and flowering, but these two responses could not be separated. However, bud cultures showed no dormancy,

and a specific effect of low temperature on flower induction was demonstrated. Low temperature appears to have a dual effect, releasing bud dormancy and inducing flowering. Potential flower buds have a deeper dormancy than vegetative buds and the first stages of flower initiation seem to occur before the winter rest period.

Arora, (1993) has been reported the self incompatible in pummelo, sweet lime and lemon. Naurial, (1972) reported better fruit set by cross than by self or open pollination in pummelos indication self - incompatibility. In sweet lime, maximum set was recorded with the pollen of Duncan grapefruit (Shinde and Dhuria, 1960. Kumar *et al.*, 1976).

Species of citrus have 2n = 18 chromosomes. Rough lemon and Rangpur lime have normal meiosis with nine bivalents forming at metaphase - I, whereas, Karna Khatta shows 0 - 2 trivalents per pollen mother cells. The pollen fertility is variable among the citrus species. Self and cross incompatibility is present in citrus. Cultivars of *Citrus grandis* are self incompatible and so also the hybrid cultivars. Hybrids between self incompatible cultivars are also self-incompatible and some of them are cross- incompatible (Agarwal, 1993).

Guerra *et al.,* (1997) analyzed the mitotic chromosomes of 51 accessions from Cruz das Almas, BA, Brazil. The sample included representatives of 20 citrus species, one of Poncrius and seven hybrids. All accessions showed 2n = 18 without any numerical variation. The most clearly variable karyotype feature was the number and position of secondary constrictions (SECs). In 19 accessions the SECs were not identified mainly due to the degree of chromatin condensation. In the remainder they varied in number from one to three per karyotype. They were found in the proximal region of one of three largest chromosome pairs, in the terminal / sub terminal region of smaller chromosome or more seldom, terminally in large chromosome. The high variability of karyotype feature may be due to the activation of this region in the previous interphase but may also indicate a high structural variability and heterozygosity of citrus germplasm.

Miyoshi *et al.,* (1997) found that the normal anthers in citrus are bright yellow when mature, owing to the pollen which they contain. In varieties with very defective pollen development, the anther color may be considerably lighter, whereas anthers containing no pollen are pale cream or white and usually do not dehisce. Pollen

diameter of different lemon varieties varies from 34 - 36 microns (Frost and Soost, 1968). Pollen germination in orange and mandarin ranged from 15% - 42%.

Burns, (1998) stated that fruit drop, leaf drop and flower drop have same genetic mechanisms in *Citrus* and it occurs in well defined zones called abscission zones. Abscission in citrus always in stress condition and it is called physiological drop. He also mentioned that fruit drop in Kinnow is inherited from the parental cultivar Willow Leaf as this phenomenon is genetically controlled and is more in high density plantation system in which they grow the plants.

Befu *et al.,* (2000) investigated the chromosome samples of young leaves of Tosa-Buntan' pummelo (*Citrus grandis* [L.] Osb.), 'Washington' Navel orange (*Citrus sinensis* [L.] Osb.) and Trifoliate orange (*Poncrius trifoliata* [L.] Raf.) were prepared by the enzymatic maceration method. The preparations were sequential stained with Giemsa, Quinicrine mustard (QM), Chromomycin A_3 (CMA), and 4 – 6 – diamidino - 2- phenylindole (DAPI). The chromosome lengths and banding patterns among 4 staining methods were compared. The discrimination of centromeres was easier by QM staining than by Giemsa staining. Five characteristic banding patterns were observed by CMA staining. They concluded that chromosomes samples prepared by using young leaves and QM and CMA staining are effective means to reveal chromosomes in citrus. Hence, a good possibility of karyotyping citrus in the future exists.

The flowering period of pummelo varied from January to February and the harvesting period varied from September to October depending on the maturity of fruits. Number of fruits per plants ranged from 3 - 71 with an average of 20. The fruit weight varied from 450 g to 1296 g with a mean of 820 g. Highest yield was recorded in CG-015 (64.82 kg) and maximum total soluble solids (TSS) were found in CG -021 (12.5%). The total acid ranged from 0.25 to 1.47 with average of 0.85 (Anon, 2001).

Kelli *et al.,* (2002) mentioned that the amount and quality of pollen produced by a flower is an important component of fitness. Pollen quality is often equated to pollen viability, i.e., the proportion of pollen grains that are viable. While viability can be measured in a number of ways, a common method to assess both pollen load and

pollen viability is by staining and direct count (Barrett, 1985, Dudash, 1991, Willis, 1999). Anthers are collected and suspended in a solution that contains a dye such as aniline blue. Viable or potentially viable grains absorb the dye while nonviable grains do not. The pollen solution is then dispersed onto a hemacytometer slide and the number of stained (viable) and unstained (nonviable) grains are counted using a microscope (Kearns and Inouye, 1993).

Saleem *et al.,* (2005) studied the fruit set and drop patterns in two commercial mandarin (*Citrus reticulata*) cultivars (Kinnow & Feutrell's Early) under Faisalabad conditions in response to different types and doses of fertilizer. Engro compound fertilizer (17: 17: 17) was compared with simple fertilizer applications. Type of fertilizer had no effect on fruit setting as highest fruit set (169.75 fruitlets branch -1) was recorded in Kinnow and Feutrell's Early (518.88 frutilets branch -1) trees, when fertilized with simple and compound fertilizers, respectively. Treatment with compound fertilizer @ 3 kg (in two splits) resulted in minimum total fruit drop both in Kinnow (87.16%) and Feutrell's early (96.62%). Fruit set was more (399.63) in Feutrell's Early compared with Kinnow (122.78). Fruit drop up to June in Kinnow and Feutrell's Early were 83.59% and 96.36%, respectively. Feutrell's Early retained 2.44% fruit in comparison to 10.81% in Kinnow mandarin until October, as the picking time of Feutrell's Early is earlier than that of Kinnow.

Malik *et al.*, (2006) described that *Citrus Indica* and *C. macroptera* are the wild endangered species of *Citrus* occurring in north eastern India. Surveys were undertaken in this region for ascertaining distribution, studying variability and for collection of germplasm of these two species. *C. indica*, an endemic species of this region, was collected from the Citrus Gene Sanctuary located in buffer zone of Nokrek Biosphere Reserve in the Garo hills of Meghalaya. In addition, a putative natural hybrid of *C. indica* and *C. limon* was collected for the first time from the south Garo hills. *C. macroptera* had much wider distribution and was collected from Mizoram and Meghalaya states. In Jantia hills of Meghalaya, natural populations of this species are in a highly threatened state. The two species were unevenly distributed all over the explored territory. Morphological characterization of leaves, fruits and seeds indicated the presence of sizable variability within collected accessions of these two *Citrus* species. Indigenous technical knowledge gathered

on the use and socio-economic importance indicated commercial potential for these two species in north eastern India. However, lack of cultivation of these species and clearing of forest cover at an alarming rate has led to an urgent need to adopt complementary conservation strategies to safeguard these species and to ensure their availability for future utilization. A major emphasis on developing methods for their propagation, multiplication and regeneration in *in situ* and *ex situ* conditions is required.

Woodford, (2008) descrived that Satkara tree is densely foliaged, leaf large (size blade 4.5 - 7.2 cm long, 3 - 4.5 cm broad), ptiole winged usually large or larger than blade (size 4.5 - 9.5 cm long and 2.5 - 4.5 cm broad), bisexual flowers and some with abortive pistil, rarely solitary but usually cymose. Calyx tube medium coriaceous ,deltoid, petals 4 - 5 and white, oval shaped, size is 8 - 11 mm long and 6 - 8mm broad. Stamens 23 - 30 (usually 27 - 29), filaments free and 5 – 8 mm long, anther 2-3 mm long, pistil stud-shaped,ovary 2 – 3 mm high and 4 – 5 mm diameter,13 -15 locules, style very short. Fruit is almost globular size 0.5 - 8.5 cm ht and 7.5 - 10.5 cm diameter, wt 10 - 16 oz. (283.5 g - 453.6 g), color yellow, surface almost smooth but pitted. Rind (flavedo) thickness usually 6-8mm, mesocarp (albedo) white and spongy having 6 – 8 mm thickness at the center, segments 13 -15 (usualley 14) adherence of carpels strong, vescicles plenty of juicy, 3 - 5 oz. per fruit, very sour in taste, color very light greenish white, seeds many 20 - 40 per fruit, testa creamy white, cotyledon white.

Begum *et al.,* (2009) mentioned that vitamin C occurs in different concentrations in a variety of natural samples. A variety of citrus fruit are available in Bangladesh and these are a good source of vitamin C. In Sylhet, *Citrus macroptera* (Shatkara) is one of the renowned locally available citrus fruit. *Citrus macroptera* (Shatkara) belongs to the genus citrus and has leaves 10 -12 cm long and edible fruit as large as sweet oranges. In Bangladesh it is available only in Sylhet region due to the different properties of soil and climate from the rest of the country. Shatkara is a well known fruit in Sylhet for its extensive use. It is used in curries with both fish and meat and also used to make pickles.

XueHu *et al.,* (2009) conducted the studies in Japan to elucidate the flowering, fruit drop patterns and fruit set effects of Tankan (*Citrus tankan*) and how they relate to tree productivity. Tankan had flower types that range from those that have no new leaves subtending them (leafless flowers) to those that have up to 7 leaves. In terms of flowering, leafless flowers and flowers having up to 3 leaves flowered much earlier than flowers having 4 or more leaves. Two flower/fruit drop peaks were observed. More flowers were abscised during the first peak which held in April to mid-May. The results also illustrated the importance of leafy flowers in ensuring high fruit set and productivity.

Das and Kumar, (2010) reported that Satkara (*Citrus macroptera*) is found in the north eastern regions of Shella and Dowki near Cherrapunji in Meghalaya, the Jampui Hills of Tripura and Mizoram, and the Chendel District of Manipur. Locally, it is called Satkara because 'sat' refers to multiples of seven and the fruit generally contains fourteen segments. The fruit is used in the preparation of pickles and its oil is used in the perfume industry. In January 2007, in Behliangchhip, Jampui Hills, Tripura, India, they noticed seven Satkara trees of 26 showing leaf mottling and yellowing in young shoots, symptoms typical of huanglongbing (HLB). HLB is a destructive citrus disease caused by a nonculturable, phloem-limited alpha-proteobacterium of the genus '*Candidatus* Liberibacter'.

Kishore *et al.,* (2010) studied the floral biology of purple, yellow, giant and *Passiflora foetida* at the ICAR Research Complex, Mizoram Centre, Kolasib, Mizoram, India during 2005 - 07. Purple, giant and *P. foetida* had major bloom during March-April, July - August and September - October. While major bloom in yellow was mainly during May - June and September - October. Purple, giant and *P. foetida* had the maximum duration of bloom of 42.4, 22.5 and 32.6 days, respectively during March - April with the maximum duration of effective bloom of 12.5, 8.6 and 10.4 days in purple, giant and *P. foetida,* respectively. Yellow had the maximum duration of bloom for 28.4 days and effective bloom of 10.5 days during May - June. Most of the flowers of purple (54.5%) and giant (58.5%) opened between 6 - 7 hrs, while the maximum per cent of anthesis in yellow (70%) took place between 12 - 13 hrs. Pollen dehiscence and pollination in purple and giant mainly occurred between 7 - 8 hrs, while 13 -14 hrs was the major period of pollen dehiscence and pollination in

yellow. The earliest anthesis (5 -6 hrs), anther dehiscence (6 - 7 hrs) and pollination (6 - 7 hrs) were recorded in *P. foetida*. The maximum stigma receptivity was recorded on the day of anthesis in all the passion fruits. Completely curved style was more common in all passion fruits that gave the maximum fruit set. The maximum number of bees observed between 7 - 8 hrs in purple and giant and between 13 - 14 hrs in yellow.

Shan-han *et al.,* (2010) observed that navel oranges tend to drop fruitlets excessively, and require fruit drop prevention measures to achieve satisfactory yields. Young fruitlet drop of summer gold navel orange trees were investigated during 2008 and 2009.The results showed that the peak of flower drop occurred 4 - 5 days following the initiation of flower drop and the exact peak dates varied from year to year with mid April in 2009 and late march in 2008. The first physiological fruit drop started on April 21, and lasted for about 35 days and ended at the end of May with the peak fruitlets drop occurring from end of April to early May. The second physiological fruit drop appeared in the early of May, 15 -16 days after flower drop, with the peak in the middle of May. Fruit drop duration varied from year to year. The number of flower drop accounted for 60% of the total flower and fruitlet drops, followed by the first physiological fruit drop, and that of the second physiological fruit drop was the least, accounting for 3.0%.The final fruit set rate of summer gold naval orange was 1.65%.The rates for navel yellowing and fruit splitting were 29.4% and 2.4%, respectively.

Part – II : Phytochemistry

Citrus are one of the leading fruit crops of the world due to its biochemical properties. It is well known that citrus are rich in vitamin C, beside this vitamin it also contain organic acids and minerals. All foods for humans must provide some life sustaining elements. These elements are the nutrients that supply energy after being metabolized in the body. Other qualifications for foods are its physiological and social effects. Many foods are eaten as a habit, custom or tradition, but all foods must possess acceptable

physical attributes, i.e. color and texture and desirable taste and palatability. Satkara possess all the characteristics of the foods.

Brine shrimp bioassay is a rapid general bioassay for the bioactive compound of the natural and synthetic origin (Meyer *et.al.*, 1982 and Persone, 1980). Bioactive compounds are almost toxic in higher doses. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and pharmacological activities of natural products. By this method, natural product extract, fraction as well as the pure compounds can be tested their activity.

Oil present in the peel (outer rind) of citrus fruits are used for flavoring purposes, citric acids found in the juices of fruits may be used as natural cosmetics and also for external application as a hair rinse, skin lotion and as a mouth wash (Randhawa & Srivastava,1986).

Different species of citrus fruits have a different chemical composition. While in the sweet group the principal constituent of edible portions are sugars (glucose ,sucrose) and acids (primarily citric acids and little of malic acids). The fruits of acid groups primarily contain the acids in the juice. The rinds of citrus contain pectin and essential oil. The vitamin C in the fruit juices of different species of citrus have been recorded to variable and 25 mg to 85 mg / 100 g (Ghosh, 1990).

According to McLaughlin, (1990), brine shrimp lethality bioassay is a bench top bioassay indicative of cytotoxicity and a wide range of pharmacological activities such as antimicrobial, antviral, pesticidal and antitumor activity of the compounds.

Steinmetz and Potter, (1991) reported that naturally occurring compounds found in plants have a wide range of physiological effects and may help to protect against various chronic diseases, including cancer and heart disease. The wide variety and number of known phytochemicals continue to grow, as does understanding of their role and importance in the diet. Several classes of phytochemicals, including monoterpenes, limonoids, flavonoids, carotenoids and hydroxycinnamic acid, have been isolated from citrus.

Puri *et al.,* (1996) discussed the different facets of the subject of bitterness and debitterness of citrus juices. For this purpose, the tone is set up by giving an account

of the biochemical properties of naringin, responsible for "immediate" bitterness, and of limonin, responsible for "delayed" bitterness. Their structures are elucidated, and enzymes participating in their catabolic path ways are assigned. Thereafter, methods used to determine bitterness, based on the principles of colorimetry, chromatography, and biochemistry are discussed, including their advantages and disadvantages. Finally, different physicochemical and biotechnological approaches for debittering are discussed.

Guthrie and Carrol, (1998) stated that animal studies have confirmed the ability of the flavonoids present in orange juice to prevent the development of breast cancer. Laboratory studies on human cells have supported this anticancer property of the orange juice flavonoids.

Harats *et al.,* (1998) mentioned that, as an antioxidant vitamin C can prevent the cell damage done by "free radical" molecules as they oxidize protein, fatty acids and deoxyribonucleic acid (DNA) in the body. Free radical damage has been implicated in the progression of several diverse and important disease states including cancer, cardiovascular disease and cataract formation. Being a good source of antioxidants, if regularly consumed, citrus can be an important part of a diet aimed at reducing the risk of such chronic disease

Along with vitamin C, citrus like other whole foods contain notable essential nutrients, like glycaemic and non-glycaemic carbohydrate (sugars and fiber), potassium, folate, calcium, thiamin, niacin, vitamin B_6, phosphorus, magnesium, copper, riboflavin, pantothenic acid and a variety of phytochemicals. Being a plant food it contains no cholesterol, fat or sodium. Citrus fruits are important among populations who need to overcome and prevent micronutrient deficiencies as well as those concerned with problems of over nutrition, obesity and diet related chronic diseases (Economos,1998).

Whitney and Rolfes, (1999) described that potassium is an essential mineral that works to maintain the body's water and acid balance. As an important electrolyte, it plays a role in transmitting nerve impulses to muscles, in muscle contraction and in the maintenance of normal blood pressure. The daily requirement of potassium is approximately 200mg and, while frank deficiency of potassium is rare, there is some concern that a high sodium-to-potassium intake ratio may be a risk factor for chronic

disease. Increased consumption of citrus fruits and juices is a good means of increasing potassium intake. One medium orange and one glass (225 ml) of orange juice provide approximately 235 mg and 500 mg of potassium, respectively

Whitney and Rolfes, (1999) stated that citrus is most commonly thought of as a good source of vitamin C. However, like most other whole foods, citrus fruits also contain an impressive list of other essential nutrients including both glycaemic and non-glycaemic carbohydrate (sugars and fiber), potassium, folate, calcium, thiamin, niacin, vitamin B_6, phosphorus, magnesium, copper, riboflavin, pantothenic acid and a variety of phytochemicals. In addition, citrus contains no fat or sodium and, being a plant food, no cholesterol. The average energy value of fresh citrus is also low which can be very important for consumers concerned about putting on excess body weight. For example a medium orange contains 60 to 80 kcal, a grapefruit 90 kcal and a tablespoon (15 ml) of lemon juice only 4 kcal.

Belletti *et al.,* (2004) assess the antimicrobial activity of nine different industrial essences used in a soft drink factory in relation to their composition, as well as to verify the role of vapor pressure on their bioactivity. The essences were tested against a *Saccharomyces cerevisiae* strain isolated from spoiled soft drinks. The tests were carried out by adding the essences directly to a liquid medium or into the headspace of closed systems inoculated with the yeast. The headspace composition was evaluated through a solid has microextraction–gas chromatography technique. The use of a mass spectrometer allowed the identification of the peaks detected. The microbial growth was indirectly monitored by measuring the metabolic CO_2 released by the yeast. The results obtained indicated that the most effective essences were characterized by the highest concentration of some terpenes, such as citral, β - pinene, and p - cymene. Moreover, all of the essences were more bioactive when added directly to the liquid medium.

The peel of citrus fruits is a rich source of flavonones and polymethoxylated flavones, which are very rare in other plants (Ahamad *et al.,* 2006). These compounds not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of application in the food and pharmaceutical industries. Naringin and hesperidin have many biological activities such as antioxidant antimutagenic effect, analgesic, antiinflamatory etc.

Lemon, lime and sudachi juices were tested for antibacterial activity against seven strains of *Vibrio* species (Tomotake *et al.,* 2006). All juices were effective in inhibiting the growth of the *Vibrio* strains. Citric acid, the major organic acid in these juices, was found to be responsible for inhibiting the growth of *Vibrio parahaemolyticus*. Sauce prepared from sudachi juice showed a strong bactericidal activity against *Vibrio parahaemolyticus,* whereas the sauce adjusted to higher pH values had no bacterial activity. Diluted sudachi juice or citric acid solution also had antibacterial activity independently. These results suggest that citrus fruit juices are effective in preventing infection with *Vibrio* species.

For detoxification of the body, humans need fiber (dietary or crude fiber) in their diet, one such fiber rich fruit among citrus fruits is lemon and is highly efficient when consumed raw. In a study conducted to know the benefits of lemon on disorders such as cholera, the antibacterial property of lemon was proven for Vibrio species of bacteria, which are the main causative organism for cholera (Tomotake *et al.,* 2006)..

A determination of minerals was carried out and the experimental results showed that some beneficial elements to humans such as calcium, magnesium, zinc, manganese, copper and sodium in grapefruit were abundant (Mandadi *et al.*, 2007).

Mandadi *et al.,* (2007) reported that Red Mexican grapefruit seeds are rich in antioxidants and other compounds known as limonoids. The limonoids present in citrus fruits such as grape fruits have been shown to inhibit the growth of cancer cells in cancers of the colon, mouth, lung, stomach and breast across animal and cell studies

Mandadi *et al.,* (2007) evaluated the antimicrobial properties of flavonoid-rich fractions derived from bergamot peel, a byproduct from the Citrus fruit processing industry and the influence of enzymatic deglycosylation on their activity against different bacteria and yeast. Bergamot ethanolic fractions were tested against Gram-negative bacteria (*Escherichia coli, Pseudomonas putida, Salmonella enterica*), Gram-positive bacteria (*Listeria innocua, Bacillus subtilis, Staphylococcus aureus, Lactococcus lactis*) and the yeast *Saccharomyces cerevisiae*. Bergamot fractions were found to be active against all the Gram-negative bacteria tested, and their antimicrobial potency increased after enzymatic deglycosylation. The minimum inhibitory concentrations of the fractions and the pure flavonoids, neohesperidin, hesperetin (aglycone), neoeriocitrin, eriodictyol

(aglycone), naringin and naringenin (aglycone), were found to be in the range 200 to 800 microg ml(-1). The interactions between three bergamot flavanoids were also evaluated. Bergamot peel is a potential source of natural antimicrobials that are active against Gram-negative bacteria.

According to Manners, (2007) phytochemicals such as flavonoids have been believed to have anticancer properties. Other compounds known as limonoids are also present in citrus fruits which confer anticancer properties to these fruits. Further they have also been proposed as potential agents that reduce cholesterol levels and prevent viral infections. Certain chemical compounds known as limonoids have been shown to prevent the occurrence of cancers of the breast, skin, lung, mouth, stomach and colon in animal studies and laboratory tests with human cells. Limonins are considered to be highly potent in preventing the spread of cancer owing to their increased availability in our body for a prolonged duration after being absorbed.

Oleuremi *et al.,* (2007) has experimented the peels of four varieties of citrus fruits namely *Citrus sinensis* (Washington), *Citrus sinensis* (Ibadan), *Citrus limonum* and *Citrus qurantifolia* were sun-dried and milled to obtain citrus fruit peel meal (CFPM). The CFPM of each variety was subjected to phytochemical screening to detect the presence of phytonutrients. Phytochemical screening showed that each of the peels contained tannin, saponin, phytate, oxalate, flavonoids and limonene. Whereas, the variation of each of these phytonutrients between the peel meals was significantly different ($p<0.01$) except for tannin ($p>0.05$), they were at low concentrations and below the levels reported in literature to be toxic to livestock species.

Quintero *et al.,* (2007) obtained essential oil from the cortex of *C.aurantium amara* has been used to add aroma to beverages and liquors and as an ingredient to give fragrance to soaps, detergents, cosmetics and perfumes. The fruits were collected in several sites of Táchira State, Venezuela. The oil was extracted from the cortex by cold pressing. Its components were analyzed by gas chromatography with flame ionization detector and gas chromatography-mass spectrometry. The main constituents found were the following - monoterpenes (limonene 77.90%, ^{3}- pinene 3.40%, myrcene 1.81%, and trans-ocimene 1.16%). sesquiterpenes (valencene, 0.52%), aldehydes (decanal 3.51%, dodecanal, 0.36% and geranial 0.29%), alcohols (^{3}- nerolidol, 0.85% and linalool 0.89%), and nootkatone as the only ketone. The

extraction procedure can be considered as adequate since the oil obtained does not contain p-cimene, which is an indicator of oxidation of monoterpenes in citrus essential oils. Terpinene-4-ol, a product of limonene degradation, was found in traces and thus no unpleasant odor was present. The biological activity of *C. aurantium amara* essential oil against *Escherichia coli, Stapylococcus aureus* and *Pseudomonas* was determined using filter paper discs impregnated with 20 µl of essential oil placed on agar plates inoculated with these bacteria (10^7 UFC). The biological activity was evaluated after 48 hrs, being inactive against *E. coli* and *Pseudomonas* and moderately active (17 mm) against *S. aureusi.* The results obtained confirmed the traditional properties of the essential oil studied as savoring, odor, and a perfume base, as well as a natural antiseptic to inhibit *S. aureus* growth.

Taiwo *et al.,* (2007) investigated the antibacterial effects of two plant extracts, *Citrus aurantifolia* linn (lime) and *Tithonia diversifolia* poaceae (sunflower) commonly used as traditional medicines in Nigeria. The antibacterial activities of aqueous extracts of these two plants were evaluated using disk diffusion susceptibility testing on 53 fresh human bacterial pathogens isolated in Ladoke Akintola University Teaching Hospital, Osogbo, Nigeria. These isolates included 27 *Staphylococcus* sp.,15 *Escherichia coli*, 3 *Klebsiella* sp., 4 *Proteus* sp. and 4 *Pseudomonas* sp. The Mean Zone Diameter (MZD) of inhibitions to 5 µL of *Citrus aurantifolia* linn extracts are 10, 12, 11, 17 and 16 mm for *Staphylococcus* sp., *Eschrichia coli, Klebsiella* sp., *Proteus* sp. and *Pseudomonas* sp., respectively. The MZD of inhibitions to 5 µL of *Tithonia diversifolia* poaceae extract are 1, 1, 0, 8 and 0 mm and for 5 µg ciprofloxacin (control) disk are 29, 19, 20, 25 and 15 mm for these respective organisms. Forty four percent (12 of 27) of the Gram positive and 69% (18 of 26) of the Gram negative pathogens showed Zone Diameter (ZD) of inhibition = 10 mm to 5 µL extract of *Citrus aurantifolia* linn. Four percent (1 of 27) of the Gram positive and 8% (2 of 26) of the Gram negative pathogens showed ZD of inhibition = 10 mm to 5 µL of *Tithonia diversifolia* poaceae. In comparison, 93% of the Gram positive (25 of 27) and 65% (17 of 26) of Gram negative pathogens showed zone diameter of inhibition = 10 mm to 5 µg ciprofloxacin (control disk). When compared to ciprofloxacin, 5 µL extract of *Citrus aurantifolia* linn possess approximately 33% anti staphylococcal activities of 5 µg ciprofloxacin and approximately the same activity on the Gram negative bacterial isolates tested ($p = 0.2767$). However, 5 µL extract of *Tithonia*

diversifolia poaceae does not possess any appreciable antibacterial effect on any of the isolates when compared to 5µg ciprofloxacin disc (p = 0.0045) or when compared to 5 µL extract of *Citrus aurantifolia* linn (p = 0.0149). *Citrus aurantifolia* linn showed promising broad spectrum antibacterial effects on human pathogens.

Lemon has a class of organic substance called polyphenols that are present in high amounts in the lemon peel. These dietary lemon polyphenols were fed to obese experimental animals to study the effects of polyphenols on obesity. It was found from the study that polyphenols significantly suppressed different aspects of obesity such as weight gain, fat accumulation, development of hyperlipidemia, high blood glucose levels, and insulin resistance (Fukuchi *et al.,* 2008).

Nannapaneni *et al.,* (2008) reported that citrus peel contains a range of essential oils. These essential oils are found to inhibit the growth of or kill bacteria which are pathogenic in nature. Citrus peels also possess diabetes lowering and antiperoxidative effect due to the high content of total polyphenols.

Ayoola *et al.,* (2008) stated that the volatile oil of tangerine fruit (*Citrus reticulata*) was extracted by steam distillation and assessed for antibacterial and antioxidant activity The volatile oil was tested against some Gram-negative organisms (*Escherichia coll* ATCC 35218, *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella paratyphi, Proteus mirabilis* and *Citrobacter spp*). Gram-positive organisms such *as Staphylococcus aureus* ATCC 25923, *S. aureus, Enterococcus faecalis* and a fungus (*Candida albicans*). The minimum inhibitory concentration (MIC) was determined with concentrations of oil extract ranging from 0.87 to 445 mg/ml. Result of the study showed that the oil has a broad spectrum antibacterial activity. MIC recorded were *S. aureus* (0.74 mg/ml), *S. aureus* ATTC 25923 (2.46 mg/ml), *E. faecalis* (1.26 mg/ml), *S.typhi* (2.07 mg/ml), *K. pneumoniae* (0.56 mg/ml), *E. coli* ATTC 35218 (0.19 mg/ml), *E. coli* (1.95 mg/ml), *P.aeruginosa* (0.97 mg/ml), *C. albicans* (0.68 mg/ml). Antioxidant screening with 2, 2 – diphenyl - 1- picrylhydrazyl (DPPH) was negative. Analysis of the chemical constituent by GC - MS showed the presence of D - limonene as the major constituent.

An USDA report, (2008) showed that mandarin oranges have been found to contain a wide variety of nutrients that may confer multiple benefits. It contains high amounts of

minerals such as potassium, calcium and phosphorous, while other minerals such as iron zinc, copper, and manganese are present in trace amounts. Among vitamins, vitamin C is present in the highest concentration followed by other vitamins such as vitamin A, B and E. Tangerine is also considered as a good source of fiber. The health protective properties are similar to other citrus fruits wherein both tangerine juice and tangerine peels has antioxidant, anticancer, and cholesterol reducing properties.

Citrus peels have also been noted to have antibacterial, and anti-inflammatory properties and they are also known to reduce the symptoms of disorders such as hyperthyroidism, diabetes, osteoarthritis and high blood pressure (Parmar and Kar, 2008)

Chowdhury *et al.,* (2008) have recently reported the antioxidant activity of the crude extract of *Citrus macroptera* (Satkara). They analyzed the stem bark of the plants. They also opined that no other detail works on this plant was not done till to date.

Deyhim *et al., (*2008) conducted a study on animals and found that grapefruit pulp increases antioxidant status and improves bone quality due to bone mineral deposition and by slowing down bone turnover.

Bhuiyan *et al.,* (2009) analyzed the chemical constituents of leaf and peel essential oil of *Citrus medica* L.by Gas Chromatography (GC) and Gas Chromatography and Mass Spectroscopy (GC - MS). Ninteen components accounting for 99.9% of the total oil are found in leaf oil. The major components are erucyclamide (28.43%). Limonene (18.36%) and citral (12.95%). The peel oil contains 43 components accounts for 99.8% of the total oil and the major components are isolimonene (39.37%), citral (23.12%) and limonene (21.78%).

Singh *et al.,* (2009) observed that due to increasing concerns about the development of antimicrobial resistance amongst pathogenic bacteria, alternative strategies have been sought that do not use antibiotics to reduce pathogenic bacteria from foods and patients. A natural compound that has potent antimicrobial properties in citrus peel, which contains a variety of essential oils are that inhibit the growth of or kill pathogenic bacteria. Further, the objective of their study was to verifying a possible antimicrobial activity of citrus oil. For this they had isolated the volatile oil from fresh peel of *Citrus*

sinensis collected from the market. Antibacterial activity of oil was evaluated by paper disc diffusion method against *Bacillus subtilis* and *Escherichia coll* using erythromycin as a standard and DMSO as a control sample. Zone of inhibition of *E.coli* and *B. subtilis* was 13 mm and 17 mm respectively when compared with the results of standard i.e. 31 mm against *E. coli.* and 32 mm against *B. subtilis.* Therefore in the future several citrus based phytoconstituents may be used in treating certain life threaten diseases.

The crude methanolic extract of *Dillenia indica* Linn (Dilleniaceae) leaves has been investigated by Apu *et al.,* (2010) *for* the evaluation of antimicrobial and cytotoxic activities. Organic solvent (*n*-hexane, carbon tetrachloride and chloroform) fractions of methanolic extract and methanolic fraction (aqueous) were screened for their antimicrobial activity by disc diffusion method. Besides, the fractions were screened for cytotoxic activity using brine shrimp (*Artemia salina*) lethality bioassay. Among the four fractions tested, *n*-hexane, carbon tetrachloride, and chloroform fractions showed moderate antibacterial and antifungal activity compared to standard antibiotic, kanamycin. The average zone of inhibition was ranged from 6 to 8 mm at a concentration of 400 µg/disc. But the aqueous fraction was found to be insensitive to microbial growth. Compared to vincristine sulfate (with LC_{50} of 0.52 µg/ ml), *n*-hexane and chloroform fractions demonstrated a significant cytotoxic activity (having LC_{50} of 1.94 µg/ml and 2.13 µg/ml, respectively). The LC_{50} values of the carbon tetrachloride and aqueous fraction were 4.46 µg/ml and 5.13 µg/ ml, respectively. The study confirms the moderate antimicrobial and potent cytotoxic activities of *Dillenia indica* leaves extract and therefore demands the isolation of active principles and thorough bioassay.

Hamadan *et al.,* (2010) analyzed the essential oils obtained by hydrodistillation of the fruit rinds of *Citrus jambhiri* Lush. (Rough lemon) and *C. pyriformis* Hassk (Ponderosa lemon) by capillary gas chromatography (GLC/FID) and gas chromatography–mass spectrometry (GLC/MS). A total of 94 compounds were unambiguously identified from the oils and the (hexane/ether) extracts of the rind and juices representing 98.55% and 97.98% of the total oil composition. The main component of both oils was D-limonene (92.48% and 75.56% respectively). The antioxidant, anti-inflammatory, antitrypanosomal, antimicrobial and cytotoxic activities of the essential oils were

evaluated. Whereas *Citrus jambhiri* and *C. pyriformis* have antioxidant activity with IC_{50} ± SD 37.69 ± 0.21 mg/ml and 28.91 ± 0.09 mg/ml, respectively. Ascorbic acid a known potential inhibitor for DPPH free radical an commonly used antioxidant showed an antioxidant activity with an IC_{50} value of 16.32 ± 0.16 µg/ml. Both oils inhibited the activity of 5-lipoxygenase (5-LOX) with an IC_{50} of 40 ± 1.63 and 38 ± 0.82 µg/ml, respectively, and could be considered as interesting candidates for antiinflammatory agents. The essential oils of both species showed substantial antimicrobial activity against all tested Gram positive bacteria and yeasts. The essential oil of *C. pyriformis* showed higher cytotoxic activity against tested cell lines than that of *C. jambhiri*. The IC_{50} values were 374.36 ± 43.95 µg/ml and 588.06 ± 27.12 µg/ml in case of HepG2 cells and 213.87 ± 18.50 µg/ml and 512.45 ± 61.46 µg/ml in case of MIA-PaCa-2 cells, respectively.

Part - III : *In vitro* propagation

The primary objective of this study is to develop a sustainable protocol for surface sterilization and mass production of field explants of Satkara, and microshoot grafting to improve the plant types.

Studies of *in vitro* culture of citrus tissues and organs were initiated during the last three decades (Sadhu,1986). Most investigators were restricted to nucellus tissues and embryos, but some research was also conducted of stem and other organs. The relevant literatures related to surface sterilization, *in vitro* propagation and microshoot grafting of citrus species are mentioned below.

Bonzid, (1975) cultured about 1 cm long stem segments of sweet orange, sour orange, mandarin and lemon trees in several media and found that development of shoots were affected by medium, age of cuttings, part of the stem from which it was taken and the time of year. Cuttings from mature trees rooted with difficulty but those from seedlings rooted easily.

DeLang, (1978) stated that presently Shoot Tip Grafting (STG) *in vitro* has been extensively used to recover the disease free plants from the commercial citrus varieties and become a potential tool for separating virus and virus like agents (Navarro *et al.,* 1981). This technique is based on the fact that the apical meristems contain no or little virus and meristematic cell grows faster than all viruses. Therefore, the production of disease free foundation plants by micrografting remaining the only mean to supply disease free bud sticks to the growers.

Baralass and Skene, (1982) reported that Benzyladenine at 10 µM stimulated multiple shoot production from juvenile and mature nodes of all varieties tested and induced adventitious shoots from stem internodes of seedling varieties. Multiple shoots were also produced from apical fragments of some of the citrus varieties. A hybrid variety showed the highest regenerative competence. High levels of exogenous auxin (e.g.10 µM naphthalene acetic acid, NAA) were found to be necessary for rooting *in vitro* grown shoots. The resulting plantlets were successfully transplanted to glasshouse conditions.

Edriss and Burger, (1984) used simple techniques and growth regulator treatments to improve micrografting success of 'Mexican' lime, 'Valencia' orange and 'Star Ruby' grapefruit were studied. Most scion cultivars grafted best on 'Carrizo' citrange [*Poncirus trifoliata* (L.) Rat. x *Citrus sinensis* (L.) Osbeck]. Dipping the shoot tip in 2, 4 - D or kinetin before grafting doubled the percentage of successful grafts. Highest grafting success occurred when the scion shoot-tip was placed in an inverted T incision on the epicotyl of the rootstock.

Shengeliya and Buttenko, (1986) observed the development of axillary buds of lemon cultivars (Mayer and Gruzinskii) on solid MS medium supplemented with cytokinins. The development of axiliary buds depended largely on the age of the plant (explants source) and the variety.

Chaturvedi and Sharma, (1988) obtained shoot bud formation using MS medium supplemented With BAP (0.5 mg/l) for root callus, BAP (1 mg/l) for leaf callus and BAP (2 mg/l) for stem callus of *C. jambhiri.*

Starrantino and Caruso, (1988) studied the *in vitro* propagation of the four rootstocks of citrus. The best rooting was obtained, for all the four rootstocks and also for the

"Flying Dragon" employed in the tests, utilizing the basal MS medium supplemented only with NAA 1 mg/l. The rooted young plants when 5 cm high were transplanted in small plastic containers filled with a steam sterilized mix of light volcanic soil and peat moss. After transplanting, the young plantlets were raised in a hotbed frame. Utilizing the above mentioned technique for transferring plantlets from "*in vitro*" to soil mixture, only a limited number of them were lost (2%).

Sim *et al.,* (1989) developed a micropropagation technique of *Citrus.mitis* Blanco. Different vegetative parts of *C. mitis* (C. *madurensis*) seedlings and mature plants were used as explants in culture. Shoots were readily produced from epicotyl, leaf, shoot tip and nodal segments of seedling explants. A high frequency (66 – 100%) of shoot regeneration was obtained from shoot tip and nodal stem segments of mature plants cultured on medium with or without benzylaminopurine (BAP) [benzyladenine]. Regenerated shoots were rooted in basal medium and the plantlets were successfully transplanted to soil.

Juarej *et al.,* (1990) in their experiment showed Arizona 861-S1 citron (*Citrus medica* L) infected with a severe exocortis isolate containing four citrus viroids was used as source of tissue for shoot tip grafting *in vitro.* Out of 51 attempts, 25 successful grafts were obtained. Only 16 survived plants were transplanted and of these 12 were viroid free. The viroids profile of the other four plants showed fewer viroids than the original field source. The significance of these results, as compared with previous reports on the recovery of viroid free plants, is discussed. The results showed the usefulness of shoot tip grafting *in vitro* as a tool to recover single viroids isolates from field sources.

Marin and Duran-vila, (1991) developed a tissue culture system using explants from juvenile plants of the sweet orange (C. *senesis* cv. *pineapple*). It consisted of (1) establishment of primary cultures from nodal stem segments followed by recovery of plants *in vitro* and (2) successive cycles of secondary culture consisting of the culture of nodal stem segments and recovery of whole plantlets.

Otoni and Teixeira, (1991) used the nodal segments of *Citrus. sinensis* L. Osb.cv. pera for their experiment of clonal propagation. Shoot proliferation of axillary buds cultured on MS medium was influenced by both type and concentration of cytokinin

used, benzyladenine (BA) being superior to 2ip, Zeatin and Kinetin. The optimal cytokinin concentration was 0.75 mg BA/l, yielding approximately 3.5 shoots per axillary bud. Shoot length was grater with BA, 2ip or Zeatin than Kinetin.

Amin and Akhtar, (1993) reported some findings of tissue culture of pummelo (*Citrus grandis* L) seedlings. They observed proliferation of axillay shoots from different *in vitro* grown seedlings explants when cultured on MS medium with half strength of major salts and different concentrations of BA and Kn. Shoot proliferation efficiency of the cotyledonary node explants was 5 - 7 times greater than that of nodal and shoot tip explants. At the end of three consecutive sub cultures (4 week each) 30 - 50 shoots could be harvested from each cotyledonary node explant when cultured on medium supplemented with 1 – 2 mg/l of BA. I*n vitro* proliferated >1 cm long shoots were rooted on half MS medium with both major and minor salts, and 0.1 - 0.6 mg/l of either IAA, IBA or NAA included or omitted, best rooting (85%) being achieved on the medium fortified with 0.4 mg/l of IBA.

Porntip, (1993) obtained numerous plantlets from stem tissue culture of lime (*C. aurantifolia* Swing). He used stem explant from both 5 years growing plants and 2 - 4 months old *in vitro* grown seedlings. He found similar response to tissue culture. These explants were subjected to MS nutrient medium containing various combinations of NAA, IAA and 2,4-D versus BAP, Kn and GA_3 ranged from 0.0 - 2.5 mg/l. These shoots were then transferred to MS medium containing 0.1 mg/l GA_3 for the completion of shoot and root growth.

Grosser, (1994) mentioned that citrus are generally propagated through bud grafting, cutting or layering. Propagation is therefore, limited to the period when buds are available. These conventional techniques are also not free from risk of perpetuating inborn pathogens. However, *in vitro* micrografting / micropropagation techniques can overcome the some constraints to citrus improvement and cultivation and can increase fruit quality and resistance to diseases and environmental stresses.

Singh *et al.,* (1994) reported *in vitro* propagation of *C. reticulata* Blanco and C. *limon* Barm f. Multiple shoots were obtained from shoot tips (2 to 3 mm) derived from mature plants (5 to 6 years old) of *Citrus reticulata* Blanco cv. Khasi mandarin and *C. limon* Burm. f. cv. Assam lemon when cultured of Murashige and Skoog (MS) medium, supplemented with 1.0 mg/l BAP, o.5 mg/l Kn, and 0.5 mg/l NAA. Root

induction was observed when 7- week - old single shoots (approximately 2 cm long) of both citrus species were cultured on MS medium supplemented with 0.25 mg/l BAP, 0.5 mg/l NAA and 0.5 mg/l IBA these plantlets were successfully established in the soil.

Belarmino and Posas, (1999) established a system for *in vitro* propagation of pummelo using shoot tip and single node stem segment taken from young seedlings. The procedure involved the initiation of shoots from explant tissue, followed by shoot proliferation, *in vitro* rooting, and finally, hardening of plantlets and potting out in the soil. The MS medium supplemented with 0.2 mg/l BAP and 0.5 mg/l IBA was optimum for shoot initiation from both explants. Eight week old shoots efficiently produced roots in MS medium containing 0.5 to 1.0 mg/l IBA compared with 4 week old shoots, indicating the importance of an adequate shoot growth prior to root induction.

Kumarin and Singh, (2000) in their experiment showed that when rough lemon rootstocks were cultured *in vitro* from germinating seeds and decapitated before being grafted with shoot tips of Nagpur mandarin. Both rootstocks and scions were dipped in growth regulator solutions for 10 min just prior to grafting. The solutions tested were 0.5, 1.0, 2.0 or 5.0 mg BA/l, 0.5, 1.0, 2.0 or 5.0 mg kinetin/l or 1.0, 2.0, 5.0 or 10.0 mg 2,4-D/l. Grafting success was highest (66.67%) with 1.0 mg kinetin or 10.0 mg 2,4 - D/l. Survival was increased if *in vitro* grafts were double grafted on rough lemon rootstocks rather than being transferred directly to the field.

A system of *in vitro* clonal propagation has been developed in C*itrus grandis* L by Begum *et al.,* (2001). Half strength MS medium supplemented with various concentrations of BAP and Kn was used to induce shoot formation from nodal and shoot tip explants of mature pummelo trees. Shoot induction percentage was high in 1.0 mg/l BAP. In same concentration of BAP, multiple shoots in the second and third subcultures increased six - eight times, respectively. Regenerated shoots were rooted in the same basal half strength MS medium containing 0.1 mg/l NAA. About 90% of rooted plantlets were established in the field after they were hardened.

Chaturvedi *et al.,* (2001) stated that plants of *C. aurantifolia* and *C. sinensis* raised from shoot meristem and micrografting were grown in a net house and those from

nodal stem segments in the field along with the *in vitro*-raised plants of rootstocks, namely, *C. jambhiri, C. karna* and *C. limonia*. All the plants showed normal healthy growth. Significantly enough, the meristem regenerated plants of *C. aurantifolia* attained the reproductive phase just in 1 year of transplantation to soil similar to those raised from nodal stem segments of mature trees, which also produced normal fruits in the subsequent year while growing under field conditions.

Biotechnology has played an outstanding role in boosting the citrus industry, in Spain, which is now the biggest exporter of citrus fruit with the application of micrografting (Chaturvedi *et al.,* 2001).

Hassanen and Azooz, (2003) observed that germination of citrus seeds was better on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm benzylaminopurine (BAP) than in a soil. Shoot cuttings obtained from germinated seeds were sub cultured on B5 medium supplemented with 1 mg dm_{-3} BAP, 0.5 mg dm_{-3} kinetin (Kn) and 0.5 mg dm_{-3} naphthalene acetic acid (NAA), where shoots grew and multiplied. They were rooted on half strength MS medium supplemented with 0.25 mg dm_{-3} BAP, 0.5 mg dm_{-3} NAA and 1 mg dm_{-3} isobutyric acid (IBA). Rooting under light was better than under dark. Seedlings and shoot cuttings with roots were transferred successfully to the soil after three weeks of acclimatization.

Singh *et al.,* (2003) studied the comperative growth of the micro propagated plantlets and seedlings of citrus varieties. They used twelve important citrus cultivars mostly indigenous to north east regions of India. Growth of tissue culture derived plantlets was compared with the seedling of the age of 60 days and at one year. In general, the initial growth rate of the seedlings was more vigorous than micro propagated plantlets but later micro-propagated plantlets became robust. However, at the age of one year micro-propagated plantlets performed better than seedling. Micro propagated plantlets had significantly higher plant height, root length, number of leaves and number of secondary roots and plant weight than seedling.

Tangolar *et al.,* (2003) in their study found that shoot tips with apical meristems and 1 - 2 leaf primordial of early Cardinal and Yaloba incise were grafted on the cut surface of the hypocotyls of Dogridge rootstocks under aseptic conditions. The survival rates of different grafting combinations were varied.

Usman *et al.,* (2005) explored citrus cultivars for multiple shoot induction and root regeneration in different media. The multiple root and shoot induction was found directly proportionate to the increase in the levels of Benzyle aminopurine (BA) and naphthalene acetic acid (NAA) in the modified Murashige and Skoog medium. The study might be promising towards *in vitro* propagation of sanitated citrus plant material.

Altaf, N., (2006) investigated that tissues from field grown trees have contamination problems in *In vitro* cultures. Two hundred explants were tried in each sterilizing chemical viz., $HgCl_2$ and $Na(OCl)_2$. Both the chemicals were effective in making clean explants growth as 62 (31%) and 67 (33%) respectively. The dead explants were 25 and 23. The buds sprouted and upon sub culturing, shoots were grown in MS medium supplemented with BA (2 mg/l), GA (1 mg/l), proline (5 mg/l). The developed shoots rooted in half strength medium with addition of IBA (2 mg/l). Our objective was to obtain rooted shoots. Although there were callus formation, leaf growth but no true shoot elongation. The rooted shoots were grafted on young rough lemon seedlings. The overall procedure is lengthy but plants can be obtained from *in vitro* bud culture of field grown Kinnow tree.

Ali *et al.,* (2007) carried out the potential uses and applicability of micrografting technique for the development of virus free nursery in citrus. Some techniques that tend to increase the grafting success were employed. MS media added with 3%, 5% and 7% sugar was used in two grafting methods e.g. inverted T incersion and surface placement on Kinnow mandarin and Succari sweet orange. The grafting was carried out under aseptic conditions by using 15 days etiolated seedlings of rough lemon. Shoot tips (1-2 mm) and leaf primordial (0.3 – 0.5 mm) were taken from the fresh shoot flashes and grafted *in vitro.* Higher grafting success of 34% was recorded in case of inverted T incersion than surface placement which give 26.7% successful. A total of 21% micro grafts were achieved at 3% sugar level which increased significantly to 33% with increase in sugar level to 5% in both cultivars. Overall, Kinnow mandarin showed relatively better.

Fifaei, *et al.,* (2007) in their experiment in Washington Navel Orange used shoot tip grafting *in vitro* is used to recovery of pathogen free plants. This study was carried out for producing Citrus Tristeza Virus (CTV) free plants. Shoot tip of infected

Washington Navel orange by tristeza containing meristem and two leaves primordial was carried. Then, it was grafted on Troyer citrange and Citronello seedlings by two methods, inverted T budding and in contact with the vascular ring. These seedlings had 3 - 5cm length. Grafted seedlings were planted on liquid medium and incubated in growth chamber at 27^0C and 16 hrs photoperiopd with 1000 lux illumination. The successful grated plants were transplanted in to the soil.

Sharma *et al.,* (2007) in their experiment on Kinnow (a citrus hybrid) and obtained Indian Ring Spot Citrus Virus (IRSCV) free plants through microshoot grafting. Nodal segments from infected mother plant were cultured on MS medium supplemented with different concentration of antiviral chemicals and growth media like 2ip (1 mg/l) and malt extract (800 mg/l). Shoot tips of size 0.7 mm were excised from the sprouts of nodal segments and grafted on to rough lemon (*Citrus jambhiri* L) under aseptic condition.

Miah *et al.,* (2008) established a protocol for direct multiple shoot regeneration from both *in vitro* grown seedlings and mature plants of *Citrus macroptera*. Both nodal and shoot tip explants taken from *in vitro* grown seedlings were cultured in MS supplemented with different concentrations of BAP and Kn either singly or in combinations. Both these explants are capable to regenerate and produce *in vitro* multiple shoots. Maximum number of shoots was obtained from nodal explants in MS supplemented with 1 mg/l BAP. BAP alone was found superior than Kn. On the other hand, only nodal explants from mature plants were used and 1 mg/1 BAP was also found best suitable for shoot induction and multiplication. *Ex vitro* rooting in pot soil (mixed with biogas slurry derived from cow-dung) was most successful compared to *in vitro* rooting in half strength of MS supplemented with different concentrations of NAA and IBA.

Perez *et al.,* (2010) investigated the influence of the basal medium and different plant growth regulators on micropropagation of nodal explants from mature trees of lemon cultivars. Although the basal medium did not affect any of the variables, explants on DKW medium were greener. Several combinations of 6-benzyladenine (BA) and Gibberellic Acid (GA_3) were used to optimize the proliferation phase. The number of shoots was dependent on the BA and GA_3 concentrations and the best results were obtained with 2 mg l^{-1} BA and 1 or 2 mg l^{-1} GA_3. Explants length was

shorter with the higher BA concentrations and, in all genotypes, shoot length was greater with 2 mg l^{-1} GA_3.The best results for productivity (number of shoots × the average shoot length) were obtained with 2 mg l^{-1} BA and 2 mg l^{-1} GA_3, although explants with chlorosis and narrow leaves were observed. The presence of BA and GA_3 in the proliferation medium was essential for the explant multiplication but GA_3 had a greater influence. The transfer of *in vitro* shoots to rooting media, containing different concentrations of Indole - 3 Butyric Acid (IBA) and Indole Acetic Acid (IAA) produced complete plantlets. Lemon shoots rooted well in all rooting combinations. The highest rooting percentages were obtained on media containing 3 mg l^{-1} IBA alone or IBA in combination with 1 mg l^{-1} IAA and on these media the highest numbers of roots were produced. The average root length was affected significantly by the IBA and IAA concentrations. Root length was greater when only 3 mg l^{-1} IBA was used, and in this rooting medium explants had a better appearance, with greener and larger leaves. The success during the acclimatization was close to 100% and the plantlets exhibited normal growth in soil under greenhouse conditions.

Samarina *et al.,* (2010) found that the effect of sterilization agents and the composition of the nutrient medium on lemon explants regeneration, micropropagation and rooting was investigated. Nodal segments of eight year old lemon trees cv. Novoaphonsky, Beskolyuchy, Udarnik, Meyer lemon were taken as explants during spring. Veltolen 0.2%, 0.2% $HgCl_2$ and hypochlorite Ca 7% solutions were used as disinfectants at different exposures. The best results for surface sterilization was obtained when shoots were deepen in Veltolen solution by about 40 min. In this case 58.7% sterile explants were received. Several combinations of BAP with NAA were used for optimization of cell proliferation and micropropagation. Maximum effect at the stage of proliferation was obtained by adding to the MS basal medium 0.1 mg/l BAP and 0.5 mg/l NAA culture media consisted of MS minerals and vitamins with 1 mg/l BAP and 0.1 mg/l NAA was stimulated multiplication of regenerated plants. Rooting of regenerants was obtained on $^1/_2$ MS supplemented with 0.5 mg/l NAA alone.

Sen and Dhawan, (2010) described a complete micropropagation protocol employing node segments from branches of adult trees of 'Swingle' citronelo. Multiple shoots were induced *in vitro* through enhanced axillary branching. A multiplication rate of 3.79 - fold every four weeks was achieved on Murashige and Skoog's media (1962) supplemented with cytokinins (6 - benzyl amino purine and kinetin) with 4% sucrose and 0.4% agar gel. The highest percent rooting was observed with a combination of indole-3-acetic acid and indole-3-butyric acid. The hardening survival rate of the rooted plantlets was 100% in a soil and agropeat combination.

Aazami and Pouraghdam, (2010) studied the shoot tip micrografting of some Iranian grapevine cultivars under *in vitro* conditions. Shoot tip explants of four cultivars were grafted on *in vitro* derived rootstocks of 4IB cultivar. The results showed that *in vitro* derived shoot tips had higher (40.3 – 60.9%) successful graft union composed to *in vivo* grown shoot tips. There was significance difference between cultivars regarding successful graft union and subsequent growth related traits. Shoot and root related growth characteristics of *in vivo* growth grafted shoot tips had some advantages in contrast with *in vitro* grown explants. In conclusion, literatures study suggested that micrografting is an alternative suitable propagation method leading the higher growth potential of grafted populations.

PURPOSE OF THE STUDY

Purpose of the study

Citrus macroptera Mont. Var. *annamensis* (Satkara) is a minor citrus fruit of Bangladesh. This fruit is consumed by the people of greater Sylhet division of Bangladesh and a certain amount of fruits are being exported to the ethnic markets of different countries. The tasty pickles made with Satkara are now available in chain shops in the different cities of Bangladesh and are also being exported. The production of Satkara in our country is not sufficient to meet our present demand. As a result, large percentage of our demand is being meet by importing fruits from neighboring states of India.

Considering the above observations the present study has been undertaken with the following objectives:

Biology

1. To study the morphological characteristics of plant and yield potentials of Satkara germplasm.
2. To study the floral characteristics includes floral biology, pollen viability, stigma receptivity and pollination behaviour.
3. To study the flower and fruit drop.
4. To study different stages of flower bud and fruit development and
5. To study the physical characteristics of fruits and
6. To study the mitotic cell of root tip cells.

Phytochemistry and biological evaluation

To determine and evaluate the followings:

1. The proximate values of the fruits.
2. The pH, Total Soluble Solids (TSS) of both rind and juice.
3. The ascorbic acid (Vit-C), other organic acids of juice and rinds of fruit.
4. The micronutrients present in rind and juice.
5. The phytochemical components present in the essential oil.
6. The antioxidant activity of the rind extracts.
7. The cytotoxicity/ brine shrimp lethality bioassay of the extracts and
8. Antimicrobial activity of the essential oil.

In vitro propagation

The main objectives of the present *in vitro* propagation are as follows,

1. To develop a suitable protocol for the surface sterilization of field explants for *in vitro* regeneration from stem buds.
2. *In vitro* germination of pummelo rootstock and (*In vitro*) micrografting.
3. *Ex vitro* shoot tip grafting (STG) of Satkara explants.
4. Successful growth of grafted plants *in vivo.*
5. *In vitro* regeneration of shoots from nodal explants of mature field plants.
6. Ex vitro rooting of regenerated shoots and
7. Transplantation of rooted plantlets.

PART - I

BIOLOGY

Chapter - 1

Materials and Methods

BIOLOGY

Materials and Methods

The experiments under the present study were conducted on ten randomly selected Satkara germplasm of 13 - 14 years old at the orchard of Citrus Research station BARI, Jaintapur, Sylhet during the period from 2009 to 2011.

Materials

1. The ten germplasm of Satkara labeled as CM -001, CM -002, C -003, C -004, C -005, CM -006, CM -007, CM -008, CM -009, CM -010 were selected in this study. Flowers, fruits also studied from these plants. For cytological study, seedling were grown in Botanical garden of University of Dhaka.

2. Measuring tape, measuring scale, slide calipers, nylon net, poly bag, transparent poly bag, paper tag, electronic microscope (Hund), slide, cover slip, filter paper, Blue Violet (BV) filter cassette.

3. Distilled water, 3% acetocarmine dye, agar, sugar, 8 -hydroxyquinoline, Acetic acid, concentrated hydrochloric acid, aceto-orcein, McIlvaine's buffer (pH 7.0) , Distamycin -A, $MgSO_4$, Chloromycin A_3 (CMA), 50% glycerol .

Methods

A) Characterization of ten germplasm

Collection of Data

1. Plant Characteristics

1.1 Tree height (m)

Tree height was recorded from ground level to height last point of canopy measured with bamboo in meters.

1.2 Base girth (cm)

The girth is measured by measuring tape at the ground level of the plant.

1.3 Shape of tree crown

Determined by standard chart and expressed as (IPGRI,1999).

i) Spheroid ii) Irregular

1.4 Tree growth habit

Recorded in the natural state a month before flowering

i) Erect ii) Spreading,

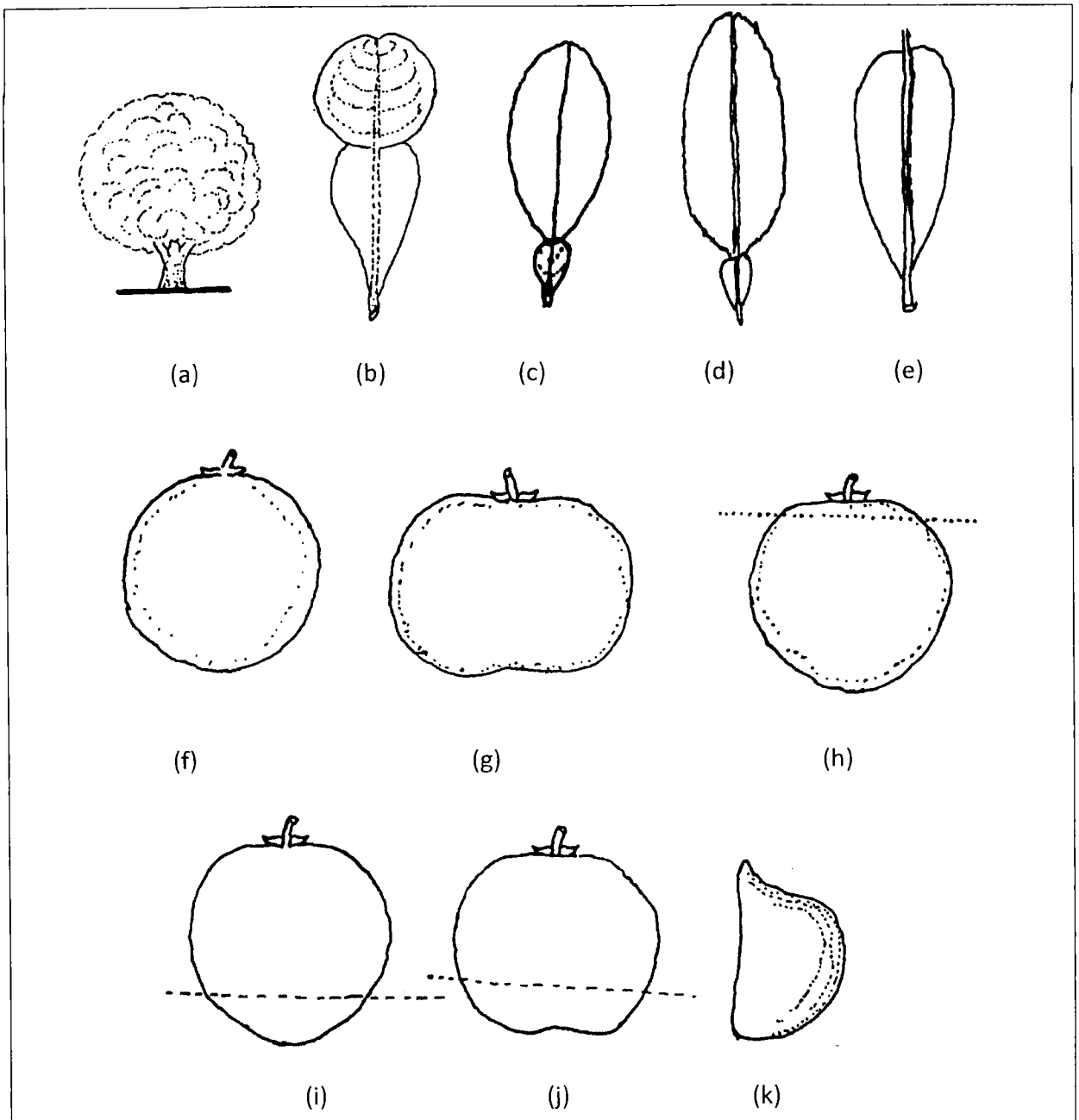

Fig: 1 The shape of the tree (canopy), leaf, fruit and seed (a) spreading shape (b) longipetiolate (c) ovate (d) crenate margin (e) obovate (f) spheroid (g) obloid (h) truncate (i) rounded (j) depressed (k) Semi-deltoid.

1.5 Density of branches

Assessed by visual observation

i) Low ii) medium iii) dense.

1.6 Thorniness

Spine density on adult tree by visual observation

i) Low ii) medium iii) high.

1.7 Tree vigor

Tree vigor was estimated by visual observation and expressed as

i) Low ii) medium iii) high.

1.8 Canopy diameter (meter)

It was measured by a measuring tape at the widest position.

2. Leaf characters

Ten fully expanded leaves were collected from each germplasm.

2.1 Shoot tip color

i) Green ii) light green iii) yellowish green.

2.2 Leaf lamina attachment

Length of petiole relative with leaf lamina

i) Brevipetiolate (petiole shoter than lamina)
ii) Longipetiolate (petiole longer than lamina)

2.3 Leaf lamina length (cm)

Recorded from base of the petiole to lamina top.

2.4 Leaf lamina width (cm)

Recorded at the widest point of leaf blade.

2.5 Petiole length (cm)

Recorded from leaf base to leaf lamina attachment was recorded by scale.

2.6 Lamina petiole ratio

Length of petiole was divided by average length of lamina.

2.7 Petiole wing shape

From the visual observation of different mature leaves of different plant.

i) Obovate

2.8 Leaf apex

From the visual observation

i) Rounded

2.9 Leaf lamina margin

From the visual observation of different mature leaves of different plant.

i) Crenate.

2.10 Leaf lamina shape

Assessed by visual observation

i) Ovate

3 Flower Characters

Data of different parts of flower were recorded from 5 flowers of each germplasm at full opening stage and mean value was calculated.

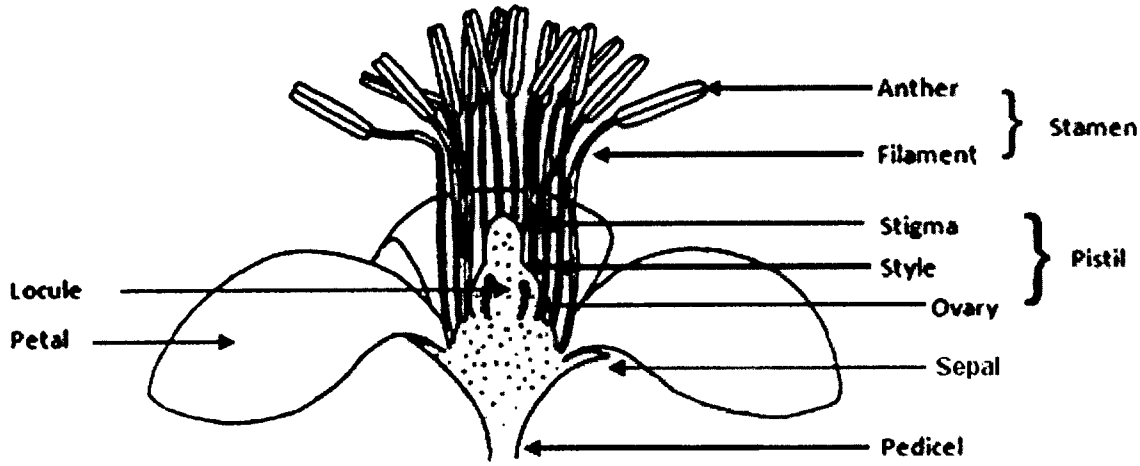

Fig: 2 Longitudinal section of a flower (diagrammatic) of Satkara germplasm.

3.1 Start date of flowering season

The different germplasm were visited frequently and time of flower initiation was recorded.

3.2 End date of flowering season

The different germplasm were visited frequently and ending time of flowering was recorded.

3.3 Flowering month

The different germplasm were visited frequently and maximum date of flowering were recorded.

3.4 Arrangement of flowers

Flowers in a tree of different germplasm were observed and arrangement was recorded.

3.5 Flower type

Five randomly selected flowers were observed from each germplasm

3.6 Color of unopened flower/bud

Recorded by visual observation of 5 flower cluster from each plant.

3.7 Color of open flower

The visual observation of 10 randomly selected flowers of different germplasm.

3.8 Pedicel length (cm)

Pedicel length was recorded from lowest point of the pedicel to the base of the calyx.

3.9 Calyx width (cm)

Diameter was recorded at the widest point of the calyx by a slide caliper.

3. 10 Number of sepals

Sepals were separated from the previously selected flowers and data were recorded by counting the numbers

3.11 Sepal length (mm)

Recoded from the base to tip of the sepal

3.12 Number of petals per flower

Petals were separated from the flowers and data were recorded by counting the numbers.

3.13 Petal length (cm)

Recorded from base to tip of the petal

3.14 Pistil length (mm)

Recorded from base of the receptacle to the tip of stigma

3.15 Style length

Recorded length between the upper side of the ovary and base of a stigma

3.16 Length of ovary (mm)

It was measured by a slide calipers from 10 different flowers and mean value was recorded.

3.17 Diameter of ovary (mm)

It was measured by a slide calipers from 10 different flowers and mean value was recorded.

3.18 Number of locules per ovary

The dissection of ovary was made by sharp blade and placed on a cleaned slide under compound microscope. Number of locules were recorded by counting from different flower.

3.19 Stamen numbers

The number of stamens were recorded by counting of 10 randomly selected flowers and mean value was recorded.

3.20 Stamen length (cm)

Recorded from the base to the top of the stamen.

3.21 Diameter of stigma (mm)

Diameter of stigma was measured by slide calipers from 10 different flowers and mean value was recorded.

3.22 Length of stamen related to pistil

Position of stamen related to stigma whether anthers are longer or shorter than stigma was recorded

3.23 Color of anthers

Anthers colors from 10 randomly selected flowers were recorded.

3.24 Presence of rudimentary pistil

Flowers of different germplasm were observed and number of rudimentary pistils were recorded.

3.25 Pollen size (µm)

Pollen grains from freshly dehisced anther were collected. Then grains were placed in glycerine media with 3% acetocarmine in slides covered by zero power cover slips. The diameters of pollen grains were measured by stage and ocular micrometer using 45× objective and 10 × eye piece. The pollen diameters were studied by taking 300 grains of each germplasm. The diameter was measured with the following formula,

$$\text{Real size} = \frac{\text{Length on micrometer}}{\text{Magnification rate}} \times 100$$

3.26 Shape of pollen

Pollens were taken from anthers of different flowers and prepared slide with 3% acetocarmine dye on a slide and covered with cover slip and shape of pollens were observed and recorded the shape.

3.27 Viable pollen (%)

Pollens were taken from freshly dehisced anthers of different flowers and prepared slides with 3% acetocarmine dye on a slide and covered with cover slip and all the visible (colored) and nonviable (not taken dye) pollens were counted under microscope. Percentage of viable pollens were calculated by the following formula,

$$\text{Percentage of viable pollen} = \frac{\text{Stained pollen numbers}}{\text{Total number of pollens}} \times 100$$

3.28 Pollen germination (%)

Pollens were taken from freshly dehisced anthers of different flowers and placed on 5 grooved slides having media of 1% agar with 3% sugar and kept under moist condition and observe first after 24 hrs and next after 12 hrs.

$$\text{Percentage of pollen germination} = \frac{\text{Germinated pollen numbers}}{\text{Total number of pollens}} \times 100$$

4. Fruit characters

The data were recorded from well developed mature fruit of each germplasm at harvesting period.

The different characteristics of fruit were described according to descriptor for citrus (IPGRI, 1999).

4.1 Shape of fruit

Fruit shape was determined by citrus descriptor and expressed as,

1 Spheroid 2 Obloid

4.2 Shape of the fruit base

The stalk end of the fruit is considered as its base. Shape of the fruit base observed at fruit maturity as

1 Truncate 2 Other

4.3 Shape of apex

The stylar end of the fruit is considered as its apex. Shape of the fruit apex was observed at maturity as

i) Deepressed ii) rounded .

4.4 Flavedo (fruit skin) color

Recorded at maturity as

i) Light yellow ii) greenish yellow iii) light green.

4.5 Albedo (mesocarp) color

Recorded at maturity as

i) White ii) Other.

4.6 Rind Smoothness

The epicarp is called Flavedo (fruit skin) and mesocarp is called albedo. Both Flavedo and albedo unitedly called rind or rind. Surface of several fruits in the plant was observed keenly and recorded.

4.7 Bitterness of peel

The bitterness of the peel of mature fruits was tested organoleptically and recorded as follows;

i) Nil ii) low iii) medium iv) high

4.8 Juice vesicles/pulp color

The color of pulp vesicles from fruits were observed and recorded.

4.9 Taste of pulp

The sourness of the pulp of mature fruits was tested organoleptically and recorded as follows;

i) Low ii) medium iii) high iv) very high.

4.10 Juice color

The juice color of different fruits were observed and recorded.

4.11 Shape of seeds

After separation of seeds from fruits, 10 seeds were selected randomly and shape was determined by comparing with standard chart.

4.12 Color of seeds

The color of seeds were observed keenly and recorded.

4.13 Month of harvest

When the fruit color in a plant start to change or fully change from green to light green and attain maximum size, then it is supposed to be matured and ideal time for harvest. This time was recorded for each germplasm.

4.14 Fruit weight (g)

Weight of the fruits from each germplasm was taken by an electric balance and the average was recorded.

4.15 Size of the fruit (cm)

Diameter of fruit was measured by slide calipers from 3 fruits of each germplasm and mean value and range were recorded.

4.16 Peel thickness (mm)

The fruit cut into halves and then rind were separated from juicy part and thickness of peel measured in mid points and mean value was recorded.

4.17 Numbers of segments per fruit

The numbers of segments in cut halves of 10 fruits from different plants were counted manually.

4.18 Juice content (ml)

The separated pulp vesicles were blended with a blender and sieved with a fine cloth and weight of 5 different fruits of each germplasm were recorded.

4.19 Days taken from fruit set to harvest

It was recorded (days) by observing each germplasm at the time of fruit set to harvest.

4.20 Number of fruits per plant

When the fruits attain maximum size then all the fruits in a plant were counted visually and number was recorded.

4.21 Number of seeds per fruit

After separation of seeds from each fruit of different plants were counted separately and mean value were recorded.

B) Flower and fruit drop

Methods

A flowering branch of stem was selected at the peak flowering time and covered by nylon net bag and counted the numbers of buds and open flowers and expressed as percentage. During 2009, the percentage was counted from 10 plants but during 2011 the percentage was counted from 7 plants because there was no flower in the rest of 3 plants. The observation was done after 3 - 4 days interval till full drop of buds and flowers and initial fruit setting. Next observation was done after 15 and 30 days interval up to maturity of fruits.

C) Fruit growth and development

At the beginning of flowering the huge numbers of buds were observed and after 15 days the well developed 50 flowers were tagged. Then data were collected after 20 days interval initially and after 70 days the data were taken after 30 days interval.

Collection of data

1. Sizes of the flower bud: After 15 days among the remaining healthy buds the size of the 10 buds measured randomly by slide calipers and measuring scales and expressed as mm.
2. Fruit size: After 20 days of fruit setting when all the floral parts except calyx abscised. The sizes of the 10 fruits were measured randomly by scale and slide calipers and expressed as cm.
3. Color of the fruit: Fruit color measured by visual observation as green, deep green and yellow of maximum fruits.
4. Oil glands: Oil glands of maximum fruits were observed by naked eyes.

D) Mitotic chromosome of root tip cells

Methods

Collection of root-tips (RTs)

Roots were collected from the botanical garden, Department of Botany, University of Dhaka. The young healthy roots were cut 0.5 cm away from the tip by a clean blade.

Pre-treatment

The collected roots were washed with water and pretreated with 8-hydroxyquinoline (0.002 M) for one hr at 15 -18°C.

Fixation

Roots were fixed in 45% acetic acid for 15 minutes at 4°C.

Preparation of slide for Orcein-staining

The pretreated roots were hydrolyzed for 15 sec at 60°C in a mixture of 1N HCl and 45% acetic-acid (2:1). Then the hydrolyzed roots were soaked on a filter paper and taken on a clean slide. The meristematic region was cut with a fine blade under a dissecting microscope. A drop of 1% aceto-orcein was added to the material and kept in an acetic acid chamber for 2 hrs. A clean cover glass was placed on the material. At first the materials were tapped gently by a tooth pick and then squashed by placing thumbs. During tapping and squashing care was taken so that the cover glass should not be moved because a minute displacement of it could damage the entire preparation. Thus way prepared the 5 slides and were observed in Hund Electric microscope.

Preparation of slide for fluorescent banding

For fluorescent banding, Alam and Kondo's, (1995) method was used with slight modification. After hydrolyzing and dissecting, the materials were squashed with 45% acetic acid. The cover glasses were removed quickly on dry ice and allowed to air dry for at least 48 hrs before study. The air-dried slides were first pre-incubated in McIlvaine's buffer (pH 7.0) for 30 mins followed by Distamycin A (0.1 mg/ml) treatment for 10 minutes and then rinsed mildly in McIlvaine's buffer supplemented with $MgSO_4$ (5 mM) for 15 mins. One drop of Chloromycin A_3 (CMA, 0.1mg/ml) was added to the materials for 15 mins and rinsed with McIlvaine's buffer with $MgSO_4$ for 10 minutes. Slides were mounted in 50% glycerol and kept at 4^0C for overnight before observation. These were observed under Hund Fluorescent microscope with Blue Violet (BV) filter cassette.

Chapter - 2

Results

Results

The results obtained from the present study are presented below:

A) Plant Characterization

1. Plant characters

Plant characteristics of Satkara are presented in Table 1.1 (Plate1.1 and 1.2).The trees were evergreen, average height of 13 -14 years grafted plants were 2.57 m, highest plant height was recorded in CM -004 (2.92 m) and lowest in CM -001 (2.01 m). The base girth was ranged 27 – 55 cm). Canopy shape was found both spheroid and irregular, and tree growth habit mostly erect and in some cases slightly curved due to weak root system and germplasm CM -008 has shown spreading type of canopy.

Out of ten germplasm the density of branches were low in five (CM -005, CM -006, CM -007, CM -008 and CM -010), medium in four (CM -001,CM -002, CM -004 and CM -009) and high in one (CM -003). Straight green thorns (Plate 1.3 a) were seen in early stages but very few in later, whereas in case of seed borne plants huge straight thorns seen at the juvenile stage (Plate 1.3 b), the overall spine density were low in all germplasm. Tree vigour of three germplasm were low (CM -008, CM -009 and CM -010), three were medium (CM -005, CM -006 and CM -007) and four were observed high (CM -001, CM -002, CM -003 and CM -004). Average canopy diameter was 2.89 m, the highest diameter was recorded in CM -004 (3.66 m) and lowest in CM -010 (2.03 m).

Table: 1.1 Plant characteristics and yield of ten germplasm of *Citrus macroptera* (Satkara).

Germ plasm	Tree ht (m)	Base girth (cm)	Shape of tree crown	Tree growth habit	Density of branches	Spine density	Tree vigor	Canopy Diameter (m)
CM001	2.08	34	Irregular	Erect	Medium	Low	High	3.12
CM002	2.54	40	Irregular	Erect & curved	Medium	Low	High	2.03
CM003	2.79	38	Irregular	Erect & curved	Dense	Low	High	3.43
CM004	2.92	50	Spheroid	Erect	Medium	Low	High	3.66
CM005	2.79	35	Spheroid	Erect	Low	Low	Medium	2.29
CM006	2.64	55	Spheroid	Erect	Low	Low	Medium	3.30
CM007	2.74	38	Spheroid	Erect & curved	Low	Low	Medium	3.18
CM008	2.80	27	Spheroid	Spread	Low	Low	Low	3.05
CM009	2.16	45	Irregular	Erect	Medium	Low	Low	2.78
CM010	2.23	34	Irregular	Erect	Low	Low	Low	2.03
Range	2.08 - 2.92	27 -55						2.03-3.66
Mean	2.57± 0.29	39.60± 7.89						2.89 ± 0.55

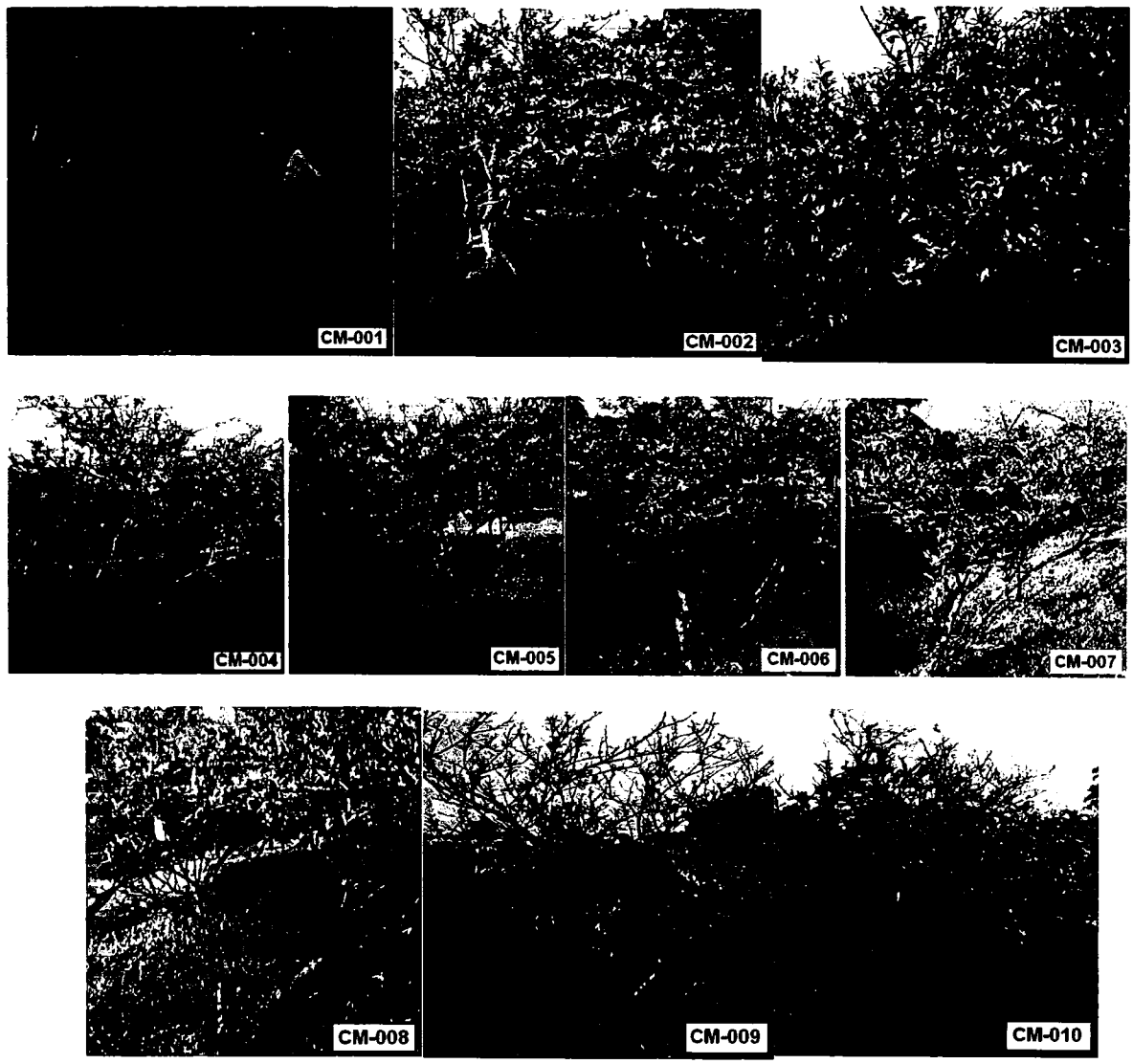

Plate:1.1 The tree (canopy) shape and growth habit of different Satkara germplasm during the first week of January, 2009.

Plate:1.2 The tree (canopy) shape and growth habit of different Satkara germplasm during the first week of January, 2011.

2. Leaf characters

The fully expanded leaves of each germplasm were collected for study.The characteristics of leaves are presented in Table 1.2 (Plate: 1.1 & plate 1.3). The dark green leaves were longipetiolate (petiole longer than the leaf lamina). Broad and winged obovate (Plate 1.1 e) shaped petiole was a typical distinguishing characteristic of the *Citrus macroptera* species. Shoot tip color was pale green (Plate 1.3 c) to light yellowish at very early stage (Plate 1.3 d). Leaf lamina and apex shape were rounded and lamina margin crenate.

Length and width of leaf lamina, petiole length and width and ratio of petiole and leaf lamina are also presented in Table 1.2. The average leaf lamina length was 6.07 cm and width 4.66 cm. Petiole was slightly longer than leaf lamina (Plate 1.3 e) and in some cases double than lamina (Plate 1.5 f) and mean length was 8.02 cm and width 5.26 cm. The lamina and petiole ratio was 1: 1:31.

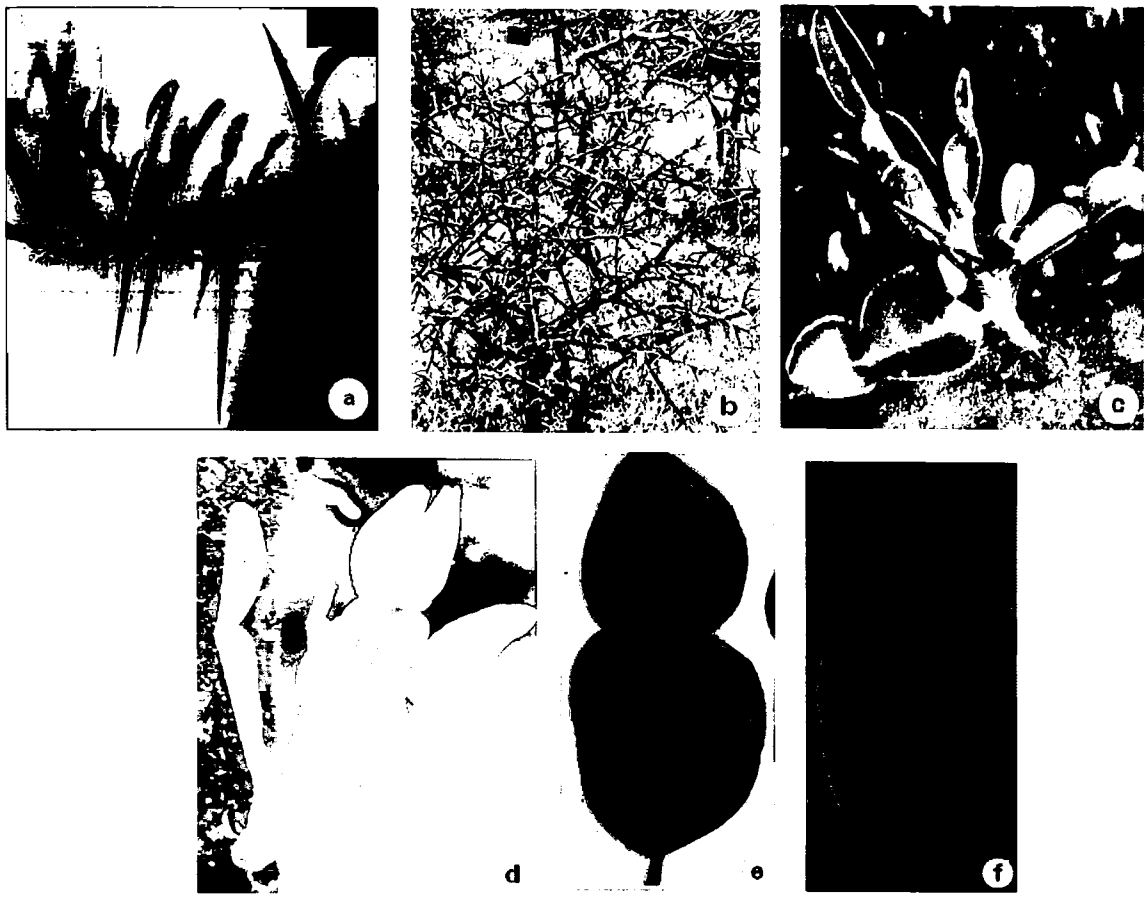

Plate: 1.3 Leaf, thorns and new shoots of Satkara germplasm (a) thorns (b) juvenile stage of seed born plant (c) young greenish shoot (d) pale yellow shoot (e) winged petiole larger than leaf lamina (f) winged petiole is double than leaf lamina

Table: 1.2 Shoot and leaf characteristics of different Satkara germplasm.

Germplasm Nos.	Shoot tip color	Leaf lamina attachment	Leaf lamina size (cm)		Petiole size (cm)		Lamina/Petiole ratio	Petiole/Wing shape	Leaf apex	Lmina/margin	Lamina/shape
			Length	width	Length	width					
CM001	Pale green	Longipetiolate	7.10	5.00	9.20	5.50	1:1.30	Obovate	Rounded	Crenate	Rounded
CM002	Pale green	Longipetiolate	5.50	4.20	7.60	5.20	1:1.38	Obovate	Rounded	Crenate	Rounded
CM003	Pale green	Longipetiolate	6.40	4.80	8.60	5.40	1:1.34	Obovate	Rounded	Crenate	Rounded
CM004	Pale green	Longipetiolate	6.50	4.80	8.50	5.40	1:1.30	Obovate	Rounded	Crenate	Rounded
CM005	Pale green	Longipetiolate	6.20	4.30	8.20	5.30	1:1.32	Obovate	Rounded	Crenate	Rounded
CM006	Pale green	Longipetiolate	5.80	4.20	7.80	5.00	1:1.35	Obovate	Rounded	Crenate	Rounded
CM007	Pale green	Longipetiolate	6.00	5.20	8.00	5.20	1:1.33	Obovate	Rounded	Crenate	Rounded
CM008	Pale green	Longipetiolate	5.50	4.80	6.80	4.80	1:1.24	Obovate	Rounded	Crenate	Rounded
CM009	Pale green	Longipetiolate	5.20	4.80	7.00	4.80	1;1.35	Obovate	Rounded	Crenate	Rounded
CM010	Pale green	Longipetiolate	6.50	4.50	8.50	4.50	1:1.31	Obovate	Rounded	Crenate	Rounded
Range			5.2-7.1	4.2-5.2	6.8-9.2	4.8-5.7	1:1.24-1:1.38				
Mean			6.07± 0.50	4.66± 0.33	8.02± 0.70	5.26± 0.28	1:1.31				

3 Flower Characters

The flower characteristics of different germplasm are presented in Tables 1.3, 1.4, and 1.5. Flower bud initiation, maximum flowering periods and end of flowering times presented in Table 1.3. From the present observation it has been found that maximum flowering occurred during 2009 but percentage were poor during 2010 and 2011 with delay flowering time. Peak flowering time of Satkara was mid February to mid March. During 2009 maximum days for flowering were recorded in CM -001 (54 days) and minimum in CM -009 (35 days), 2010 maximum in CM -004 (32 days) and minimum in CM -009 (24days) and 2011 maximum in CM -003 (30 days) and minimum in CM -003 (20 days) .

Floral arrangement, type, number and length of floral accessory parts are presented in Table 1.4. Arrangements of flowers were both solitary and clustered (Plate 1. 4 h & i) and inflorescence was cymose. Initially the bud color was purple but latter cross purple margin was shown on the top (Plate 1. 4 a, h, i), generally flowers were white at maturity (Plate 1. 4 b) but sometime purple color also found (Plate 1.4 j). The other characteristics were recorded like average pedicel length 1.28 mm, calyx diameter 2.07 mm, number of sepals usually 5 and sepal length 1.40 mm, the number of petals usually 5 but 4 and 3 was also recorded (Plate 1.4 c) and petal length 7.35 mm.

Table: 1.3 Flowering periods of different Satkara germplasm.

Germplasm Nos.	Flowering time						Flowering Month (peak time)
	Start			End			
	2009,	2010.	2011	2009,	2010.	2011	
CM-001	Jan30th	.Feb15th	, Feb22th	March25th	March18th	March 20th	February
CM-002	Feb5th	No,	Feb25th	March20th	No	March23th	February
CM-003	Feb10th	Feb22th	Feb18th ,	March17th	March20th	March19th	February
CM-004	Jan29th	Feb15th	Feb25th	March16th	March19th	March25th	February
CM-005	Feb 7th	No,	Feb22th	March20th	No	March20th	February
CM-006	Feb5th	Feb19th	Feb25th	March18th	March15th	March18th	February
CM-007	Feb4th	Feb23th	No	March15th	March22th	No	February
CM-008	Feb8th	Feb25th	Feb24th	March19th	March22th	March16th	February
CM-009	Feb10th	Feb20th	No	March17th	March16th	No	February
CM-010	Feb5th	No,	No	March15th	No	No	February

Table: 1.4 Floral arrangement, type, number and length of floral accessory parts (calyx and corolla) characteristics of different Satkara germplasm.

Germplasm Nos.	Arrangement of flowers	Flower type	Color of bud	Color of open flower	Pedicel Length (mm)	Calyx Diameter (mm)	No.of sepals /flower	Sepal Length (mm)	No.of petals /flower	Petal Length (mm)
CM001	Solitary&clustered	Bisexual	purple	White	1.50	2.42	5	1.50	5	8.20
CM002	Solitary&clustered	Bisexual	purple	White	1.30	2.20	5	1.75	5	7.50
CM003	Solitary&clustered	Bisexual	purple	purple	1.20	2.20	5	1.25	5	7.20
CM004	Solitary&clustered	Bisexual	purple	White	1.20	2.30	5	1.50	5	7.00
CM005	Solitary&clustered	Bisexual	purple	Light,purple	1.30	2.10	5	1.25	5	7.20
CM006	Solitary&clustered	Bisexual	purple	White	1.50	2.00	5	1.25	5	8.00
CM007	Solitary&clustered	Bisexual	purple	White	1.10	2.10	5	1.25	5	7.00
CM008	Solitary&clustered	Bisexual	purple	Light purple	1.20	2.00	5	1.50	5	7.20
CM009	Solitary&clustered	Bisexual	purple	White	1.20	2.10	5	1 75	5	7.00
CM010	Solitary&clustered	Bisexual	purple	White	1.30	2.10	5	1.25	5	7.20
Range					1.1-1.5	2.00-2.42	5 - 5	1.25-1.75	5 - 5	7.00-8.20
Mean					1.28± 0.3	2.07± 0.30	5± 0.0	1.40± 0.20	5 ± 0.00	7.35±0.42

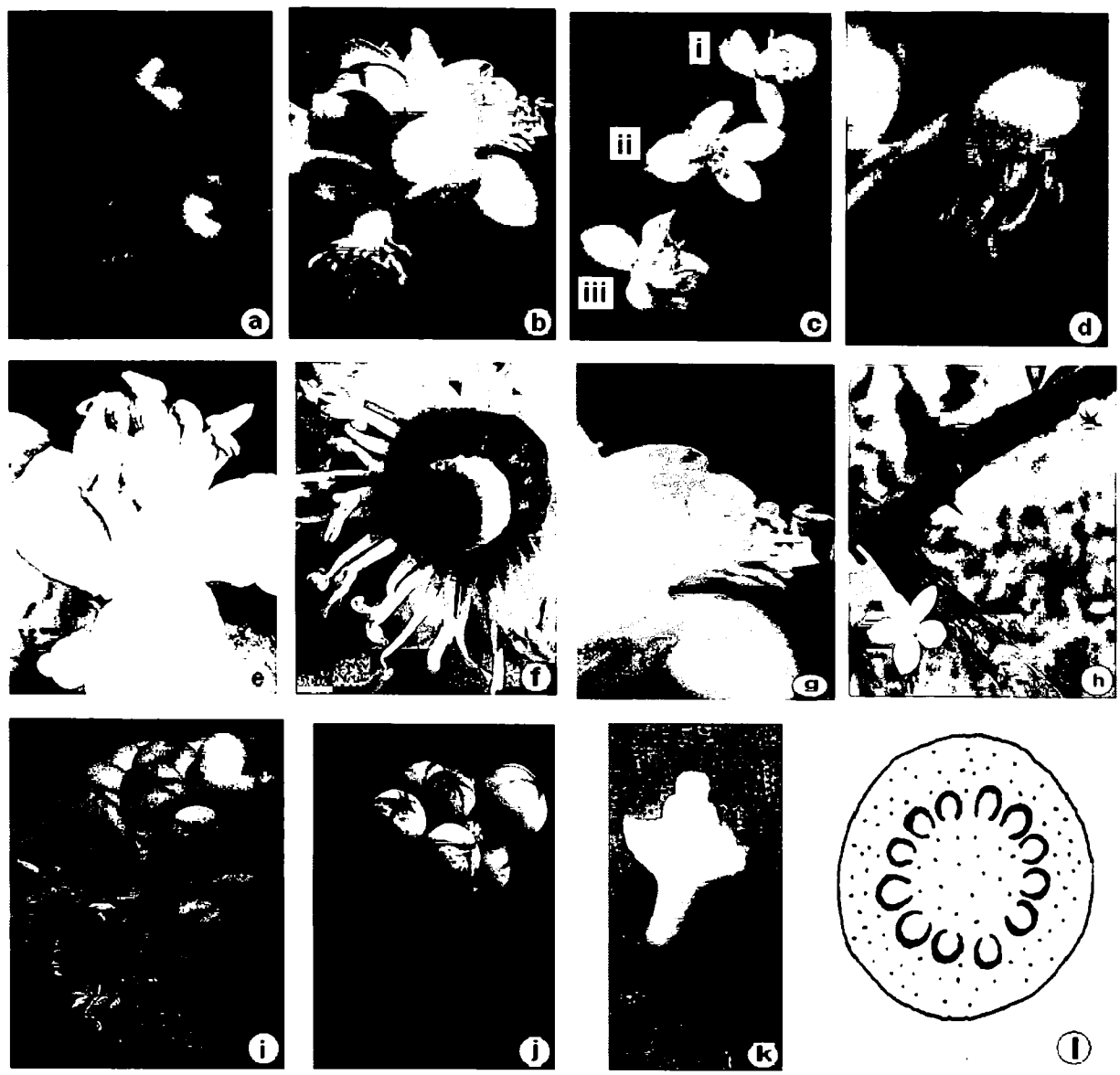

Plate: 1.4 Flower characters of Satkara germplasm (a) purple flower buds (b) open white flower (c) i) trimerous, ii) tetramerous , and iii) pentamerous flower (d) light purple flower (e) yellow anther initiatated (f) withering of anther (g) whitish anther (h) solitary flower (i) flowers in cluster (j) cymose inflorescence (k) pistil with pedicel and receptacle (l)T.S. of ovary (diagrammatic)

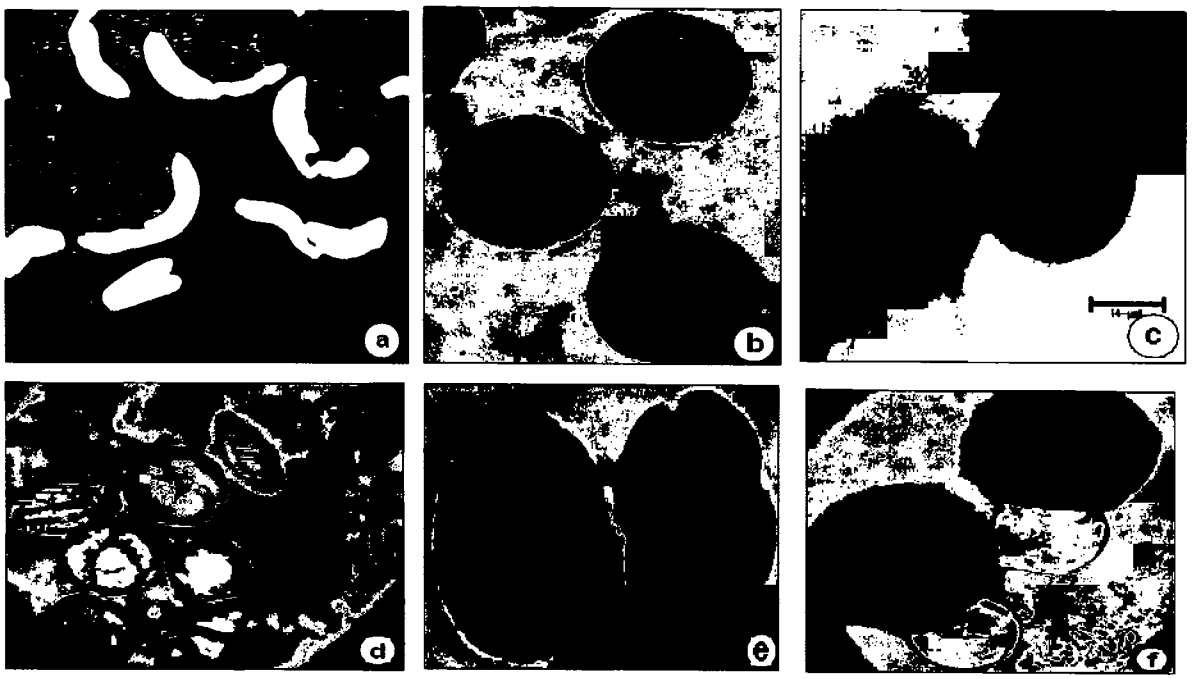

Plate:1.5 Pollen viability and pollen germination of Satkara germplasm (a) anther (b) round shape pollen (c) size of the pollen (d) viable and non viable pollen (e) germinating pollen grains at initial satge (f) pollen tube .

Table: 1.5 Characteristics of different floral parts of of Satkara germplasm.

Germplasm	Pistil length (mm)	Style length (mm)	Length of ovary (mm)	Diameter of ovary (mm)	Nos of locules per ovary	Stamen numbers	Stamen length (mm)	Stigma length (mm)	Stigma diameter (mm)	Length of stamens related to style	Anther color	Presence of rudimentary pistil (%)
CM001	3.00	1.00	1.3	2.5	14	26	26	1.5	1.8	Longer	Yellow	25
CM002	3.50	1.00	1.0	2.5	14	24	24	1.2	1.6	Longer	Yellow	30
CM003	3.50	1.00	1.2	2.3	14	25	25	1.3	1.8	Longer	Yellow	25
CM004	3.00	1.25	1.2	2.2	14	27	27	1.5	1.8	Longer	Yellow	30
CM005	3.00	1.00	1.1	2.5	14	26	26	1.5	1.6	Longer	Yellow	35
CM006	2.75	1.00	1.2	2.5	14	25	25	1.4	1.6	Longer	White	40
CM007	3.00	1.50	1.1	2.2	14	29	29	1.5	1.8	Longer	Yellow	35
CM008	2.75	1.25	1.0	2.3	14	25	25	1.4	1.6	Longer	Yellow	35
CM009	3.50	1.00	1.3	2.0	14	24	24	1.3	1.6	Longer	White	25
CM010	3.25	1.00	1.2	2.2	14	24	24	1.5	1.6	Longer	White	25
Range	2.75-3.5	1.0-1.5	1.1-1.3	2.0-2.5	14.00	24-29	5.5-6.5	1.2-1.5	1.6-1.8			25-40
Mean	3.12± 0.29	1.1± 0.17	1.16± 0.11	2.33± 0.17	14.00	25.6 ±1.57	6.05± 0.68	1.41± 0.11	1.68± 0.10			30.5± 5.50

Table: 1.6 Pollen characteristics of different Satkara germplasm

Germplasm	Shape of pollen grain	Pollen diameter (µm)	Pollen viability (%)	Pollen germination (%)
CM001	Rounded	27.50	80.00	36.60
CM002	Rounded	26.00	82.00	40.00
CM003	Rounded	25.40	75.00	30.00
CM004	Rounded	23.80	81.75	40.00
CM005	Rounded	26.50	74.75	30.66
CM006	Rounded	24.50	79.00	41.25
CM007	Rounded	27.20	75.00	30.00
CM008	Rounded	27.50	80.25	35.20
CM009	Rounded	24.00	71.55	36.0
CM010	Rounded	26.00	75.00	30.00
Range		23.80 – 27.50	71.55 – 82.00	30.00 – 41.25
Mean		25.44 ± 1.43	75.08 ± 3.40	34.97 ± 4.55

The characteristics of different floral parts of Satkara germplasm are presented in Table 1.5. The female organ pistil was small and stud shaped and length was 3.12 mm. Style was very short and fixed in the cavity of ovary and length around 1 mm. Ovary length was 1.16 mm and diameter 2.33 mm and locules per ovary 14. The stamens were free and numbers 24 - 29, stamen average length was 6 mm (filament 4 and anther length 2 mm). Stigma was capitate and length and diameter 1.41 mm ×1.68 mm. Length of stamens were longer than pistil, anther color usually yellow (Plate 1.4 e) but some time also shown pale and white color at the time of withering (Plate 1.4 f, g). The number of rudimentary pistil was recorded 25 – 40% and average 30.5%.

Pollen characteristics of different germplasm are presented in Table 1.6. Pollen shape was usually rounded ((Plate 1.5 b) and average pollen diameter was 25.44 µm. Percentage of viable pollens was 75.08 %(Plate 1.5 d). The germination (Plate 1.5 e & f) percentage of pollen was 34.97%.

4 Fruit Characters

The yield and morphological traits of fruits and seed of Satkara germplasm are presented in Table 1.7. The fruit shape were usually obloid (Plate 1.1 g) but frequently spheroid (Plate 1.1 f). Shape of the fruit base was truncate (Plate 1.1 h) and the apex were rounded and depressed (Plate 1.1 i, & j). The flavedo, surface of peel color light yellow, green yellow and light green were recorded. The albedo part was white (Plat 1.6 e), smoothness of rind surface "smooth & pitted" (Plate 1.6 c), bitterness was tested organoleptically and recorded nil, low, medium and high. The pulp color was creamy white (Plate 1.6 g) and taste highly sour. The juice color was creamy white. The seed shape semi deltoid and color was creamy (Plat 1.6 f). Fruits harvesting month was October

The quantitative characteristics of fruits and seeds of Satkara germplasm are presented in Table 1.8. The fruit weight was recorded 165-260g per fruit, the length was 4.50cm –6.00 cm and diameter 5.20 - 7.25cm. The thickness of rind was 8.20 mm. The number of segments was 14 (Plate 1.6 g). Average juice content 131 ml per fruit and fruits per plant was recorded 12.20, days required from fruit set to harvest recorded 175.55 and seeds per fruit was 6 -11.

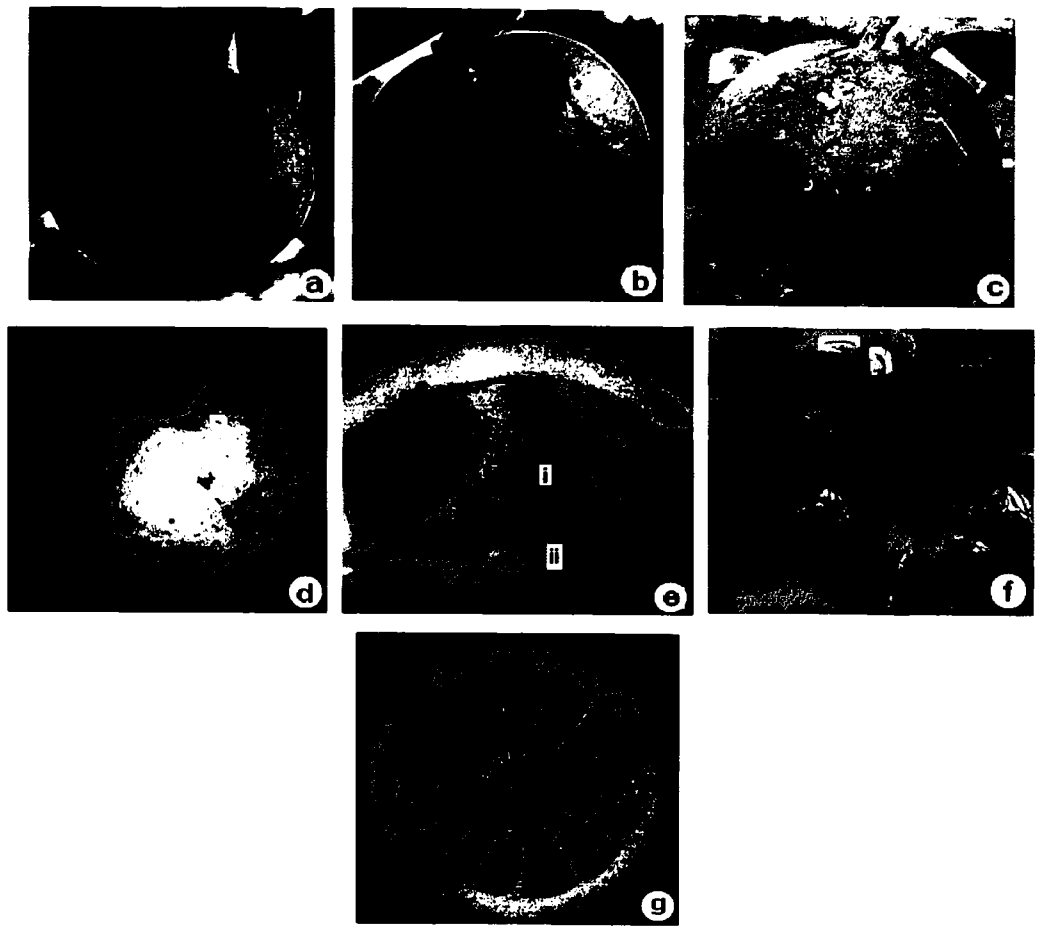

Plate: 1.6 Fruit and seeds of Satkara germplasm (a) green fruit with smooth surface,base convex, apex rounded (b) fruit base truncate (c) fruit apex is depressed (d) yellow color mature fruit (e) peel (i)flavedo, (ii)albedo (f) seeds (g)T.S. of fruit showing 14 locules.

Table: 1.7 Yield and morphological traits of fruits and seed of Satkara germplasm.

Germplasm Nos.	Fruit shape	Shape of fruit base	Shape of fruit apex	Flavedo color	Albedocolor	Rind smoothness	Bitterness of peel	Pulp color	Taste of pulp (sourness)	Juice color	Shape of seeds	Seed color	Month of harvest
CM001	Obloid	Truncate	Depressed	Yellowish green	White	Smooth & pitted	Medium	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM002	Obloid	Truncate	Depressed	Light yellow	White	Smooth & pitted	Nil	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM003	Spheroid	Truncate	Depressed & rounded	Yellowish green	White	Smooth & pitted	Low	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM004	Obloid	Truncate	Rounded	Yellowish green	White	Smooth & pitted	High	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM005	Obloid	Truncate	Rounded	Light yellow	White	Smooth & pitted	Nil	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM006	Obloid	Truncate	Depressed & rounded	Yellowish green	White	Smooth & pitted	Low	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM007	Obloid	Truncate	Depressed	Yellowish green	White	Smooth & pitted	Low	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM008	Spheroid	Truncate	Depressed	Yellowish green	White	Smooth & pitted	Low	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM009	Obloid	Truncate	Depressed & rounded	Yellowish green	White	Smooth & pitted	Nil	Creamy white	High	Cream	Semi deltoid	Creamy	October
CM010	Spheroid	Truncate	Rounded	Light yellow	White	Smooth & pitted	Medium	Creamy white	High	Cream	Semi deltoid	Creamy	October

Table: 1.8 Quantitative characteristics of fruits and seeds of Satkara germplasm.

Germplasm Nos.	Wt per fruit (g)	Fruit sizes		Thickness of rind (mm)	Nos. of segments per fruit	Juice content per fruit (ml)	No of fruits per plant	Days from fruit set to harvest	No of seeds per fruit
		Length (cm)	Diameter (cm)						
CM001	235	5.50	6.75	9	14	130	22	175	8
CM002	240	4.75	5.50	9	14	135	15	175	6
CM003	205	5.25	6.25	7	14	120	18	180	9
CM004	220	6.00	7.25	8	14	125	15	175	8
CM005	175	5.00	6.00	8	14	140	12	170	10
CM006	190	4.75	5.50	9	14	135	8	175	9
CM007	210	4.50	5.25	9	14	125	7	175	10
CM008	165	5.50	6.20	7	14	130	10	180	8
CM009	220	5.75	6.70	8	14	140	8	170	11
CM010	230	4.50	5.20	8	14	130	7	180	7
Range	165-240	4.50-6.00	5.20-7.25	7 – 9	14	120-140	7 -22	170– 180	6-11
Mean	209± 25.30	5.15± 0.53	6.07±0.70	8.20± 0.60	14	131.00	12.20 ±5.16	175.55± 3.48	8.60 ±1.51

B) Flower and fruit drop

The results of initial numbers of buds and open flowers (Plate 1.7 c), flower dropping, fruit setting and fruit dropping of Satkara in the year of 2009 and 2011 are presented in Table 1.9 and Table 1.10. All germplasm flowered abundantly during 2009 but during 2011 flowering was not occur in CM -007, CM -009 and CM -010. Number and duration of flowers were also very low in 2011. During 2009, large number of flowering was observed (Plate 1.7 a). Flower drop during 2009 (Table 1.9) ranged from 84.75% - 100% and average recorded 92.81%. Initial fruit set percentage after 21 days varied from 00% - 15.25% and average was 07.39%. Fruit set after 120 days were 00% - 03.38% and average 0.88%.

Flower drop during 2011 (Table 1.10) were 91.27% - 100% and average was 98.32%. Initial fruit set percentage after 21 days were 00% - 03.57% and average 01.56%. Fruit set after 120 days were 00% - 01.69% and average 0.24%. Due to the presence of huge rudimentary pistils (Plate: 1.7 e)f ruit setting were found low.

As the flower is protogynous and the stamen were taller than pistil and stigma became receptive and dehiscence of anther done before anthesis, undoubtedly self pollination was occur but the flying of common honey bees (Plate: 1.7 g) at the morning of the day indicated the probability of cross pollination. At present study the details of any experiment related cross pollination were not done.

Table: 1.9 Study of flower and fruit drop of Satkara germplasm in the year of 2009.

Germplasm Nos.→ / Subjects ↓	CM - 001	CM - 002	CM - 003	CM - 004	CM - 005	CM - 006	CM - 007	CM - 008	CM - 009	CM - 010	Average (%)
No. of unopened flower(buds) at the time of first observatlon(%).	86.49	86.89	91.13	83.05	94.25	89.02	92.23	89.47	93.75	88.33	89.46 ±3.50
No. of open flower at the time of first observation (%).	13.51	13.11	08.86	16.95	05.75	10.98	07.77	10.53	06.25	11.67	10.53 ±3.50
No. of drop flowers (total)%.	90.54	100	93.67	84.75	91.95	93.90	89.32	96.49	95.75	91.77	92.81 ±4.22
No. of fruit set (%) initially (after 21 days)	09.45	00.00	06.33	15.25	08.15	06.10	10.68	03.51	06.25	08.23	07.39 ±4.11
No. of fruit set (%) .(after 120 days)	01.34	00.00	01.27	03.38	00.00	01.22	00.00	O0.00	00.00	01.67	0.88 ±1.12

Table: 1.10 Study of flower and fruit drop of Satkara germplasm in the year of 2011.

Germplasm Nos.→ / Subjects ↓	CM 1	CM 2	CM 3	CM 4	CM 5	CM 6	CM 7	CM 8	CM 9	CM10	Average (%)
No. of unopened flower(buds)at the time of first. observation(%).	93.30	88.24	93.75	91.42	92.85	90.00	00.00	92.30	00.00	00.00	91.69 ±01.97
No. of open flower at the time of first.observation(%).	06.70	11.76	06.25	08.57	07.14	10.00	00.00	07.69	00.00	00.00	08.30 ±01.97
No. of drop flowers (total) %.	96.67	100.0	97.92	100.0	96.42	100.0	00.00	91.27	00.00	00.00	98.32 ±01.63
No. of fruit setting (%) initially (after 21 days).	03.38	00.00	02.08	00.00	03.57	00.00	00.00	01.92	00.00	00.00	01.56 ±01.58
No. of fruit setting (%) (after 120 days).	01.69	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.24 ±00.63

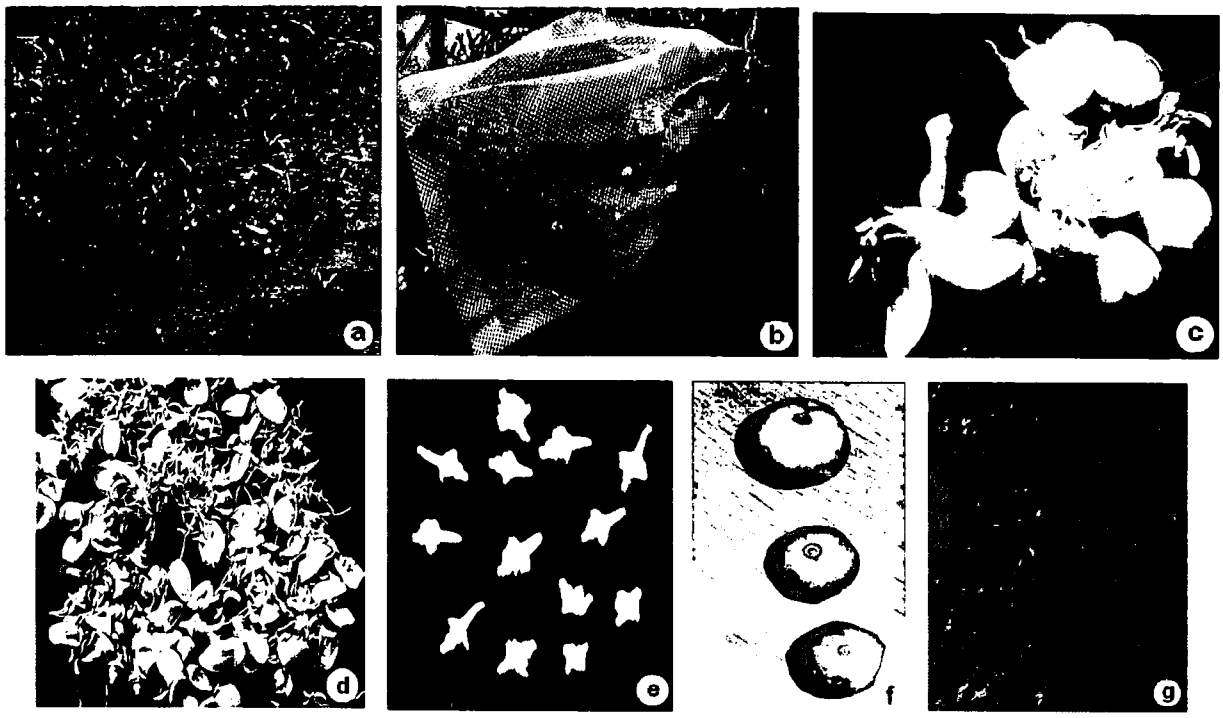

Plate: 1.7 Flower and fruit drop of Satkara germplasm (a) profuge flowers in a plant (b) flower buds covered by nylon net (c) flower cluster (d) abscised floral parts (e) rudimentary pistil (f) dropped fruitlets and g) honey bee.

C) Stages of floral bud development

The period from visible initiation of floral bud to the stage of its attaining full size (that a day prior to opening) was considered as the period of floral bud development. The floral bud development was divided into six stages (Plate 1.8). At first stage (Plate 1.8 **I**) the bud primordial was outbreak from the stem branches or from the old bark. Naturally buds bears in the last vegetative growth (branches). The stages, days, sizes and color of floral buds are presented in (Table 1.16) At the stage **I,** purple color bud primordial were seen and the length was around 1 mm at 7 days old and diameter was 2.25 mm.

After 14 days at the stage II, (Plate 1.8 **II**) the buds were at tiny stage having length 4.70 mm and diameter 5.11 mm . The color of the buds were till purple but pedicel and calyx were visible and hairy. After 21 days the bud became whitish but at the top purple cross margin remained (Plate 1.8 **III**). The length and diameter increased rapidly at this stage. The length was recorded 8.21 mm and diameter 9.00 mm which almost double than previous stage.

At the stage **IV** (28 days) the length and diameter of the buds (Plate 1.8 **IV**) increased slightly, the length and diameter were 10.10mm and 11.90mm respectively. After 35 days (stage **V**) the length was almost same as stage **IV** but diameter slightly increased. At this stage (**V**) bud reached at maximum size (Plate 1.8 **V**).The dehiscence of anther and stigma became receptive at the stage **V**, pollination, fertilization were done before the anthesis (flower petals opening). The length and diameter were 10.50 mm and 13.70 mm respectively. After this stage the flower has started to open (Plate 1.8 **VI**) and fertilized ovary (fruit) has shown with yellow stigma, white petals and yellowish stamens.

I, II, III, IV, V, VI

Plate: 1.8 Different stages of flower bud development of Satkara germplasm (**I –VI**). **I**) bud initiation stage (7 days), (**II**) very tiny stage (14 days) (**III**) medium growth stage (21 days **(IV**)mature stage (28 days) (**V**) before opening (35 days) (**VI**) fertile flower open 40 - 45 days).

Table: 1.11 Different stages of floral bud development of Satkara germplasm.

Days	Stages	Sizes of flower buds (mm)		Color	Comments
		Length	Diameter		
07	Stage - I	01.00±0.25	02.25±0.15	Purple	Attached to wood
14	Stage - II	04.70±0.42	05.11±0.22	Purple	Pedicel and calyx seen well
21	Stage - III	08.21±0.27	09.00±0.35	Purple margin with whitish petals	Diameter widen
28	Stage - IV	10.10±0.42	11.90±0.45	Purple margin with whitish petals	Some flower buds become white
35	Stage - V	10.50±0.33	13.70±0.25	Purple margin with whitish petals	At the time of anthesis
40-45	Stage - VI	---	-------	Whitish petals	Flowers already open

D) Fruit growth and development

In the present study length and diameter of fruit were recorded at 15 days intervalals to determine the growth pattern of fruit during 2009. Two germplasm were included in this study. The total growth pattern were divided into seven stages. The observation was initiated on fruit 40 days (after fruit set) from bud initiation upto 220 days when fruit attained at harvesting maturity stage. The seven stages of growth are presented in (Plate 1.9) At the stage **I,** after 10 - 12 days from initial fruit set (40 days from bud initiation), generally the light yellowish green ovary with yellow stigma was attached. Most of the fruits dropped at this stage attaining length 0.39 cm and diameter 0.27 cm. From stage **I** to stage **II,** the increament of length and diameter of fruit were very rapid. At stage **II,** the length and diameter were 1.35 cm and 2.20 cm respectively.

The length and diameter of fruit from **III** to **VII** were, at stage **III** 1.85 × 3.10 cm, at stage **IV** 2.35 × 3.90 cm, at stage **V** 3.25 × 4.85cm, at stage **VI** 4.70 × 6.20 cm and at stage **VII** 4.71 × 6.52 cm respectively.

The color of the fruit was remain deep green at the stage II and III, green at stage **IV** and **V**. At the stage VI the color of the fruit start to light green and yellowish green at stage **VII** . After harvesting or after stage VII the rind color of fruit became yellow. The color of the rind became green up to August and turn to yellow in September - October in Bangladesh. The oil glands of the rind were visible by naked eye from stage **III** and very clear up to maturity. The calyx persist up to stage **IV** and then abscised (Plate 1.9 **VI**).

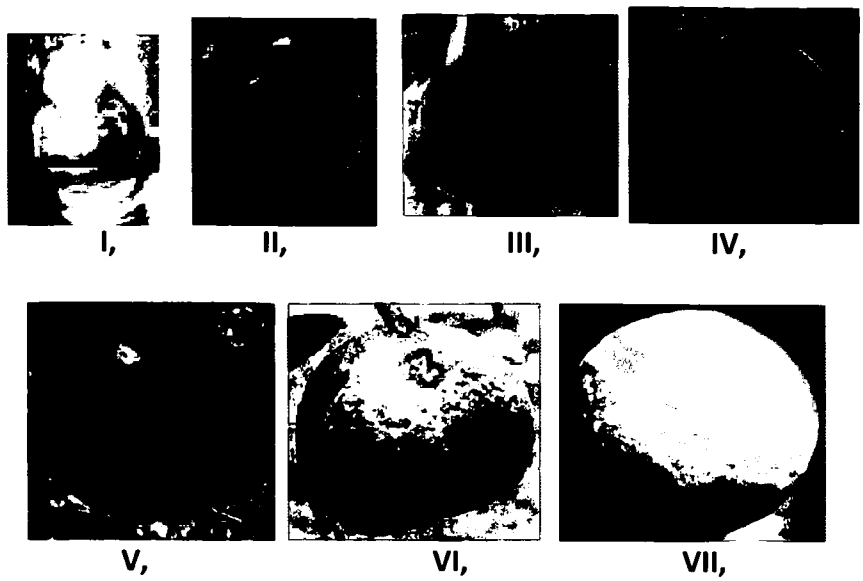

Plate: 1.9 Different stages of fruit growth and development of Satkara germplasm after 30 days interval (**I**) just after petals and stamen abscised(50 days) (**II**) 70 days from flower bud initiation, (**III**) 100 days from flower bud initiation (**IV**) 130 days from flower bud initiation (**V**) 160 days from flower bud initiation (**VI**) 190 days from flower bud initiation (**VII**) 220 days from flower bud initiation.

Table: 1.12 Growth and development stages of fruit of Satkara germplasm.

Days from bud initiation	Stages	Sizes of fruits (cm)		Color	Comments
		Length	Diameter		
40	Stage- I (Mid April)	0.39 ± 0.10	0.27 ± 0.02	Light yellow green	Stigma, stamen, petal abscised.
70	Stage- II (Early May)	1.35 ± 0.20	2.20 ± 0.25	Deep green	Almost set the fruit
100	Stage- III(Early June)	1.85 ± 0.12	3.10 ± 0.18	Deep green	Growth vigorous
130	Stage- IV Early July)	2.35 ± 0.28	3.90 ± 0.24	Green	Growth vigorous
160	Stage- V (Early Aug)	3.25 ± 0.27	4,85 ± 0.32	Green	Growth vigorous
190	Stage- VI(Early Sept)	4.70 ± 0.35	6.47 ± 0.37	Light green	Growth reduced
220	Stage- VII (Early Oct)	4.71 ± 0.32	6.52 ± 0.25	Yellowish green	Harvesting stage

E) Mitotic chromosomes

The best shown slides of different mitotic stages are placed in the Plate 1.10. The different parameters of chromosome are presented in Table 1.13. The total length of 2n chromosome complement in *Citrus macroptera* (Satkara) was 24.30 µm (Table 1.13). Individual chromosome length ranged from 0.95 to 1.89 µm. Sharp decrease of chromosomal length was not observed in this species. The relative length of each chromosome was ranging from 0.04 to 0.08 (Table 1.13). No satellite or secondary constriction was found.

The CMA - banded regions were the GC - rich repeats in the chromosomes. *Citrus macroptera* (Satkara) was found to possess many dot like CMA - positive bands (10 -15) in mitotic interphase and prophase and 8 CMA -positive bands in mitotic metaphase [(Plate 1.10 (7, 8, 9)]. Total length of CMA - positive banded region was 4.45 µm which covered 18.30 % of the total chromatin length.

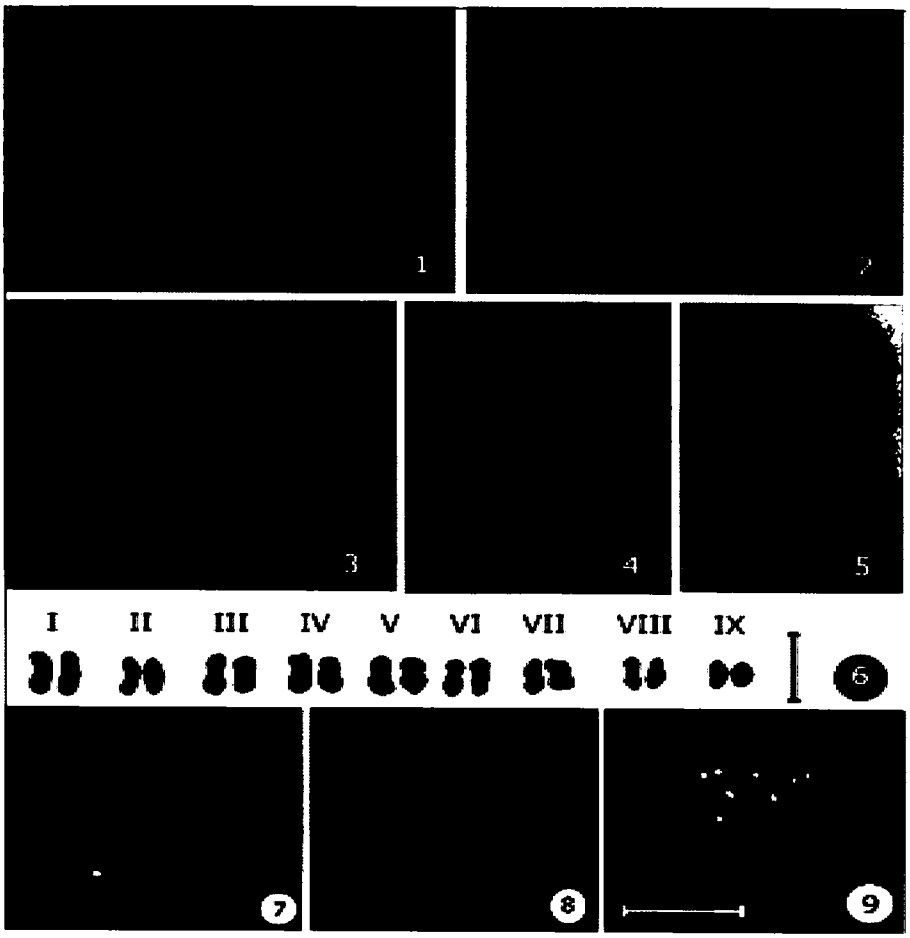

Plate: 1.10 Orcein and CMA- stained different stages of mitotic cell division of Satkara germplasm (1) orcein stained interphase nuclei (2) orcein stained prophase chromosome (3) orcein stained imetaphase chromosome (4) orcein stained anaphase chromosome (5) orcein stained telophase chromosome (6) orcein stained karyotype prepared mitotic metaphase chromosome (7) CMA - stained prophase chromosome and (9) CMA - stained metaphase chromosomes, scale bar = 10 µm

Table: 1.13 Length (µm), arm ratio, centrometric index, relative length and centrometric type of metaphase chromosome of Satkara germplasm.

Chromosome Pair	Long arm (l) µm	Short arm (s) µm	Total arm (T) µm	Arm ratio (l/s)	Relative length (R/L)	Centrometric type
I	0.95	0.94	1.89	1.01	0.08	m
	0.95	0.94	1.89	1.01	0.08	m
II	0.74	0.73	1.47	1.01	0.06	m
	0.74	0.73	1.47	1.01	0.06	m
III	0.74	0.73	1.47	1.01	0.06	m
	0.74	0.73	1.47	1.01	0.06	m
IV	0.69	0.68	1.37	1.01	0.06	m
	0.69	0.68	1.37	1.01	0.06	m
V	0.69	0.68	1.37	1.01	0.05	m
	0.69	0.68	1.37	1.01	0.05	m
VI	0.64	0.62	1.26	1.03	0.05	m
	0.64	0.62	1.26	1.03	0.05	m
VII	0.58	0.58	1.16	1.00	0.04	m
	0.58	0.58	1.16	1.00	0.04	m
VIII	0.58	0.58	1.16	1.00	0.04	m
	0.58	0.58	1.16	1.00	0.04	m
IX	0.53	0.52	1.05	1.01	0.04	m
	0.48	0.47	0.95	1.01	0.04	m

Chapter - 3

Discussion

Discussion

Plant characters

Citrus macroptera (Satkara) were grown both seed borne and grafted plants in our country. We have studied the 13 -14 years old grafted plant on pummelo rootstock. So, similarity of these plants has shown with pummelo plants mostly.

In the present study, height of the Satkara plants were recorded 2.08 m - 2.92 m and base girth 27- 55 cm. Canopy shape was found both spheroid and irregular and tree growth habit mostly erect but some plants were curve due to weak root system and CM -008 shown spread nature. The density of branches were low in six germplasm, medium (in four) and high in one. The presence of thorns observed low. Tree vigor of four germplasm were high, three were medium and two found low. Average canopy diameter was 2.89 m. Canopy diameter is an important characterestics of plant that determines the spacing.

Mondal and Amin, (1990) reported that most of the citrus plants were shrubs but few, such as pummelo became tree. Rashid, *et al.*, (1987) stated that pummelo trees were small, whereas, Purseglove, (1987) reported that height of pummelo plants ranged from 5 m to 15 m. A base girth of 13 – 77 cm recorded by Anonymous, (1999) in 48 lines of pummelo, this result fully agrees with present findings. Canopy shape was mostly irregular/ spreading nature, Hodgson, (1967) reported that pummelo plants are either spreading or dropping. Vigorous spines in young branch having 6 – 7 cm long but very few or absent in fruit bearing branches with small thorns (5 – 10 mm) in Kerr's Thailand papeda (Reuter *et al.,*1967). Hossain, (1983) observed both spiny and spineless genotypes of pummelo.

In present study the shoot tip color was observed pale green to light yellowish at very early stage (Plate 1.3 d). Leaf lamina and apex shape were rounded and lamina margin crenate. The average lamina length was 6.07 cm and width 4.66 cm. Petiole was slightly longer than leaf lamina and in some cases double than lamina

and mean length was 8.02 cm and width 5.26 cm. The lamina and petiole ratio was 1: 1:31. Winged petiolate leaf which was longipetiolate(petiole longer than lamina), petiole shape obovate and lamina shape rounded. Woodford, (2008) descrived that Satkara tree was densely foliaged, leaf large, petiole winged usually large or larger than blade. Reuter *et al.*, (1967) were reported in *Citrus macroptera* Mont.var. *kerrii* Swing that the leaf blades were sometimes ovate rounded at both ends, petiole broadly winged, obovate, slightly larger than the leaf blade, petiole 7 X 3.8 cm whereas, in case of Satkara Woodford, (2008) reported from his study that the highest leaf lamina was 7.2 X 4.5 cm and petiole 9 X 4.5 cm.

The Satkara flowers were small, bisexual and peak flowering time of Satkara was mid February to mid March. In present study flowering was started from 19th January and ended in 20th March during 2009 but flowering month is generally February. Arrangements of flowers were both solitary and clustered and inflorescence was cymose. Initially the bud color was purple but later cross purple margin was shown on the top. Generally color of open flowers generally white but exceptionally it was purple or pale in color. A mature flower bud length was 12 – 14 mm and diameter 8 – 10 mm, pedicel length very small 1 - 1.5 mm, calyx cup shaped and diameter 2 - 2.42 mm, number of sepals usually 5 but rarely 4, petals usually 5 but sometimes recorded 3 or 4 petals oval shaped and size 7.00 - 7.20 mm X 4.2-6 mm. The flower was protogynous and the stamens were longer than pistil. The pistil was small and stud shaped and length was 3.12 mm. Style was very short and fixed in the cavity of ovary and length around 1 mm. Ovary length was 1.16 mm and diameter 2.33 mm and locules per ovary 14. The stamens were free and numbers 24 - 29, stamen average length was 6.05 mm (filament 4 and anther length 2 mm). Stigma was capitates and length and diameter 1.41 mm ×1.68 mm. Length of stamens were longer than pistil and 30 - 40% rudimentary pistil were recorded. Peak flowering time of Satkara was mid February to mid March and maximum days for flowering 20 – 54. Pollen shape was usually rounded and diameter 25.44 µm. Percentage of viable pollens was 75.08% and germination 34.97%.

The characteristics of flowers and floral parts of Satkara also agreed mostly with the findings of Woodford, (2008) who descrived that Satkara is small, bisexual flowers and some with abortive pistil, rarely solitary but usually cymose. Calyx tube medium coriaceous ,deltoid, petals 4 - 5 and white, oval shaped, size is 8 -11 mm long and 6 -8 mm broad. Stamens 23 - 30 (usually 27 - 29), filaments free and 5 – 8 mm long, anther 2 -3 mm long, pistil stud-shaped, ovary 2 -3 mm high and 4 -5 mm diameter,13 -15 locules, style very short. Reuter *et al.,* (1967) reported stamens number is 16 - 20 in *C .macroptera*. Var. *kerrii.*

Woodford, (2008) stated filaments were free, length of filament was 5 – 8 mm and anthers 2 - 3 mm. Miyoshi *et al.*, (1997) found that the normal anthers in citrus were bright yellow when mature, owing to the pollen which they contain. In varieties with very defective pollen development, the anther color might be considerably lighter, whereas anthers containing no pollen were pale cream or white and usually did not dehisce. Pollen diameter of different lemon varieties were varied from 34 - 36 microns (Frost and Soost, 1968). Pollen germination in orange and mandarin ranged from 15%-42%.

Kelli *et al.*, (2002) mentioned that the amount and quality of pollen produced by a flower was an important component of fitness. Pollen quality was often equated to pollen viability, i.e., the proportion of pollen grains that were viable. Hoque, (2007) reported in pummelo germplasm that pollens were round shaped. Pollen diameter is 35 – 40 µm; viable pollen percentage is 85 - 99. Frost and Soost, (1968) reported germination percentage of orange pollens was 15% - 42% and pollen size of lemon varieties were 34 - 36 µm. These reports supported the present findings of pollen of Satkara, where round shaped pollen, size 26.67 µm, viability 81.75% and germination rate 36.66% were recorded.

At present study the fruit shape was found usually obloid but frequently spheroid, shape of the fruit base was truncate and the apex rounded and depressed. The surface of rind color was light yellow, green yellow and light green were recorded. The albedo part was white, smoothness of rind surface "smooth & pitted", bitterness was tested organoleptically and recorded nil, low, medium and high. The pulp color

was creamy white and taste highly sour. The juice color was creamy white. The seed shape semi deltoid and color was creamy .Fruits harvesting month was October.

Woodford, (2008) reported that, shape of the Satkara fruit was more or less oblate to globular, the surface color was color yellow to bright yellow, surface smoothness almost smooth but pitted, pedicel lenth small, fruit apex rounded.albedo color white and spongy, pulp vescicles white in color, jucie color liiight whitish and very sour in taste,seed shape irregular or triangular, testa creamy white. Reuter *et al.,* (1967) reported that in *C. macroptera*. var. *annamensis* fruit was globular shaped.

In present study, the fruit weight was 165 – 260 g per fruit, size 4.50 cm –6.00 cm X 5.20 -7.25 cm. Peel thickness was 8.20 mm, number of segments was 14 and juice content 131 ml per fruit. The days required from fruit set to harvest 175.55, fruits per plant was recorded 12.20, fruits harvesting month October and seeds per fruit 6 - 11.

The fruit weight and sizes were depending on the season condition, good management, irrigation and genetic inheritance of plants. Woodford , (2008) mentioned in his paper that Satkara fruit weight was 10 - 16oz. (283.5 g- 453.6 g), size 0.5 - 8.5 cm length and 7.5 -10.5 cm diameter, peel thickness 6 - 8 mm at the center, segments 13 -15 (usualley 14) ,vescicles plenty of juicy, 3-5 oz.(85 -142 g) juice per fruit, seeds many 20 - 40 per fruit, Whereas, Reuter *et al.,* (1967) reported that in *C. macroptera*. Var. *annamensis* fruit size was 4.5 X 5.2 cm. In case of pummelo (Anon, 2001) the flowering period varied from January to February and the harvesting period from September to October and the days required 210 – 230 from flowering to harvesting. Hoque, (2007) stated that pummelo required 232 days from flowering to harvesting.

Flower and fruit drop

Flower and fruit drop is a natural phenomenon which occurs in all fruit plant. Initial fruit set was found to be based on number of flowers per branch. Fruit retention

depends upon the health of the tree and later drop must be due to physiological and nutritional conditions (Saleem *et al.,* 2008).

Like other citrus species fruit drop was a serious problem in Satkara which start from blooming to fruit harvest. Flower drop were 92.81% in 2009, 98.32%.in 2011. Initial fruit set percentage after 21 days were 7.39% in 2009 and 1.56%. Final fruit set after 120 days were 0.88% in 2009 and 0.24% in 2011.

High or low temperature, rains, malnutrition, pest and diseases were various causal agents associated with the fruit drops. Fruit drop and retention are mostly related with varietal characters, although water and nutritional stress increases the fruit drop of susceptible trees (Rameshawar and Rao,1980). Nitrogen deficiencies at the time of blooming is one of the most common causes of too much fruit abscission of flowers results in less fruit (Chandler,1958).

Gonzales and Borroto, (1987) with the aim to increased citrus flowering under tropical conditions, two formulations of 2-chloroethyl phosphonic acid (Ethrel and Flordimex), Alar (succinic acid-2, 2-dimethyl hydrazide) and Cycocel (2-chloroethyl-trimethyl ammonium chloride) were applied during the flower induction period (December) to young "Valencia" orange trees at concentrations of 1000, 2500 and 5000 mg/l. These treatments effectively increased flowering, mainly at lower concentrations. Treatment using Flordimex at 500 and 1000 mg/l concentrations resulted increase of in flowering. Hossain, (1983) reported that flowering of pummelo influenced by climatic conditions

In case of Satkara, the bud stage till opening of petals required 32 - 35 days and maximum drop were done at the early bud stage. XueHu *et al.,* (2009) in *Citrus tankan* also mentioned that more abscission was done at early stage. Shan-han *et al.,* (2010) also reported in Naval orange that after 4-5 days of bud initiation maximum abscised. The final fruit setting of Satkara were recorded 0.88% in 2009 and 0.24% in 2011.This findings almost similar to sweet orange., where reported that *Citrus sinensis* may develop 2,50,000 (2.5 lacs) flowers per tree in bloom season and only a small amount of flowers less than 1% becomes mature fruits (Erickson and Brannman, 1960, Goldschmidt and Monselise,1977)

Maximum flowers (70 - 75%) dropped before anthesis and rest were dropped within 7 - 15 days. Immature fruits (10 - 30 days) drop was also high and finally less than 1% fruits were set. Bud initiation to fruit retention can be controlled by hormonal spray at different stages of citrus flowering. Application of GA_3 in *Citrus sinensis* L (Lord and Eckard, 1987), *Citrus sinensis* L and *Citrus reticulata* Blanco (Guardiola *et al.*,1982),

Burns, (1998) stated that fruit drop, leaf drop and flower drop have same genetic mechanisms in *Citrus* and it occurs in well defined zones called abscission zones. Abscission in citrus always in stress condition and it is called physiological drop. He also mentioned that fruit drop in Kinnow is inherited from the parental cultivar Willow Leaf as this phenomenon is genetically controlled and is more in high density plantation system in which they grow the plants.

Saleem *et al.,* (2005) studied the fruit set and drop patterns in two commercial mandarin (*Citrus reticulata*) cultivars (Kinnow & Feutrell's Early) under Faisalabad conditions in response to different types and doses of fertilizer. They observed type of fertilizer had no effect on fruit setting.

Shan-han *et al.,* (2010) observed that Navel oranges tend to drop fruitlets excessively, and require fruit drop prevention measures to achieve satisfactory yields. Young fruitlet drop of summer gold navel orange trees were investigated during 2008 and 2009.The results showed that the peak of flower drop occurred 4 - 5 days following the initiation of flower drop and the exact peak dates varied from year to year with mid April in 2009 and late march in 2008.The first physiological fruit drop started on April 21, and lasted for about 35 d and ended at the end of May with the peak fruitlet drop occurring from end of April to early May. The second physiological fruit drop appeared in the early of May, 15 -16 days after flower drop, with the peak in the middle of May. Duration of fruit drop varied from year to year. The number of flower drop accounted for 60% of the total flower and fruitlet drops, followed by the first physiological fruit drop, and that of the second physiological fruit drop was the least, accounting for 3.0%.The final fruit set rate of summer gold naval orange was 1.65%.

The causes of flower and fruit drop and low fruit set of Satkara are not due to abnormal environment, nutritional imbalance and other physiological factors but also due to floral abnormality like defective pollen production, anther without pollens, presence of abundant abortive pistils. The cause of abnormality occurred in many germplasm due to genetically reason also.

Stages of floral bud development

The initial stage of flower was a bud which generally outbreak from the last year grown stem in citrus plants. The growth and development of flower of Satkara, from flower bud initiation to opening of flower divided into six stages. Frost and Soost, (1968) also described the stages of reproduction in citrus lemon. They divided the stages from bud formation to fruit set into eight stages

The time between **IV** and **V** (32-35 days) is important for Satkara because dehiscence of pollens and receptivity of stigma occurred before anthesis. Although Soost (1964), supported that the dehiscence of pollens in citrus flowers usually occurs soon after the petals separate, but Hoque, (2007) reported in pummelo that dehiscence of pollens occurred in the close bud.

Time of anthesis of Satkara was observed during the study. Anthesis is very much important for breeder, as selfing must be done before anthesis. On the other hand, emasculation and bagging must be done before anthesis and receptivity of stigma. At least 3 days before of anthesis emasculation and crossing to be done, In case of Satkara, stigma became receptive before anthesis and it is confirmed by hand tasting. It has been observed in Satkara flowers the opening of petals (anthesis) start after midnight 3 hrs to 8 AM. But in some cases the unfertile flowers open frequently in the day.

At the stage of **II,** the buds were at tiny stage having 4.70 X 5.11 mm size. The color of the buds till purple, pedicel and calyx were visible, hairy with green color. After 21 days at the stage **III**, the bud became whitish but at the top purple cross margin remained. The length and diameter increased at this stage rapidly. The length was

recorded 8.21 mm and diameter 9 mm which almost double than previous stage. The anthesis and stigma became receptive at the stage **V**, fertilization was done before the flower opening. The length and diameter were 10.5 mm and 13.7 mm respectively. After this stage the flower has started to open and fertilized ovary (fruit) shown with yellow stigma.

Fruit growth and development

Fruit production is a reproductive process initiated at the time of flowering. The first stage of flower initiation is followed by flower growth, pollination and fertilization, growth of the fruit to maturity or harvest. The fruit production is highly influenced by the climatic factors. Without the effects of environmental and climatic factors, fruit production is functionally is a function of flowering and fruit setting.

Study of the fruit growth and development of *Citrus macroptera* so far not known to us, but some study has been done by several workers in other citrus species, Dhillon and Singh, (1992) in lime and mandarin Bain, (1958) in Valencia orange and Hoque, (2007) in pummelo.

In the present study the rapid growth of Satkara fruit observed primarily from mid-April to June. During June for time being the growth become slower and in citrus fruits the popular term "June drop" occurred because in this time some set fruits also dropped naturally. The vigorous growth again starts from mid July to august due to sufficient rainfall and other environmental factors. But from mid August the growth became slower and it has reduced at September. Similar findings reported by Hoque, (2007) in pummelo, Dhillon and Singh, (1993) in sweet lime, and Hittalmani *et al.,* (1977) in Tahiti lime.

The color of the fruit rind became green up to August and turn to yellowish green in September-October in Bangladesh. According to Dhillon and Singh, (1993) the cool temperature promote more rapid decomposition of chlorophylls (degreening) from

citrus rinds and subsequent development of yellow pigments, carotenoids during maturity of fruit has also been reported by Jackson, (1991).

Mitotic chromosome

The genus *Citrus* has large number of species and varieties under the family Rutaceae. Within this genus, high cross compatibility has led to natural interspecific and intergeneric hybrids resulting in numerous morphological variations. This variation has been transmitted to progenies by nucellar embryoney. Because of their heterozygosity, the taxonomic systems among citrus are still controversial (Tanaka, 1977).

The discrimination of citrus chromosome at a somatic metaphase is difficult because of their small size (1.5 µm to 3.5 µm) and similar morphology. Chromosome at pro-metaphase may be more suitable for karyotyping in citrus (Befu *et al.,* 2000). The cytological characterization of citrus species could help in the identification of a particular genomic variant or for the detection of the true hybrids in breeding programmes as well as studies of karyotype evolution of the groups (Guerra *et al.,*1997).

Karyotype is one of the most reliable parameters for identifying a taxon since it is generally a stable feature of a species. Besides this, some recently developed cytogenetical techniques may be useful to study the karyomorphology of *Citrus macropetra*. Among these techniques, DNA base-specific chromosome banding with different fluorochromes may be most suitable (Schweizer, 1976). In this technique, usually two fluorochromes, *viz.* Chromomycin A_3 (CMA) and 4- 6 Diamidino-2-phenyl Indole (DAPI) have been using widely. CMA binds with GC - (Guanine-Cytosine) rich and DAPI with AT - (Adenine- Thymine) rich repeats of the genomes, respectively. Thus it would be easier to identify even an individual chromosome with the help of differential fluorescent banding (Schweizer, 1976, Alam and Kondo, 1995, Akter and Alam 2005, Sultana and Alam , 2007).

The aim of the present work was to investigate the Karyology (shape, size, number, constriction etc) and CMA banding of Satkara chromosome for future biological and hybridization studies.

The karyotyping of citrus has been carried out with root tip cells of seedlings and they found 2n = 18 (Guerra,1993. Matsuyama *et al.*,1996 and Miranda *et al.*,1977.) The works of Guerra *et al.*, (1997) in 20 species of citrus was more extensive cytogenetic study so far carried out. All germplasm presented 2n = 18, with very similar karyotypic morphology and size, although a single seedling of *Citrus sinensis* cv Berna was triploid. They also observed secondary constriction (SECs) in some species but in present study of Satkara root tip cells no SECs was recorded.

The CMA - banded regions are the GC - rich repeats in the chromosomes (Schweizer, 1976). *Citrus macroptera* was found to possess many dot like CMA - positive bands (10 - 15) in mitotic interphase and prophase and 8 CMA-positive bands in mitotic metaphase [(Plate 1.12 (7, 8, 9)]. Total length of CMA - positive banded region was 4.45 μm which covered 18.30 % of the total chromatin length.

Chapter - 4

Conclusion

Conclusion

The biological study of different aspects of Satkara through various experiments revealed a conclusion about the different parameters of this plant, leaf, flower, fruit, flower and fruit drop, stages of bud development, growth and development of fruit and mitotic chromosomes of root tip cells.

The height of the Satkara plants were recorded 2.08 m -2.92 m, base girth 27 - 55 cm, canopy shape was both spheroid and irregular and tree growth habit mostly erect. The density of branches were generaaly low and medium , presence of thorns observed low. Tree vigor of four germplasm were high, three were medium and two found low. Average canopy diameter was 2.89 m.

The shoot tip color was found pale green to light yellowish at very early stage. Leaf lamina and apex shape were rounded and lamina margin crenate, lamina length was 6.07 cm and width 4.66 cm, petiole slightly longer than leaf lamina and in some cases double than lamina and mean length was 8.02 cm and width 5.26 cm. The lamina and petiole ratio was 1: 1:31. Winged petiolate leaf which was longipetiolate (petiole longer than lamina), petiole shape obovate and lamina shape rounded

The Satkara flowers were small, bisexual and peak flowering time of Satkara was mid February to mid March but flowering month is generally February and maximum days for flowering 20 – 54. Arrangements of flowers were both solitary and clustered and inflorescence was cymose. Initially the bud color was purple but latter cross purple margin was shown on the top. Generally color of open flowers were white but exceptionally it was purple or pale in color. A mature flower bud length was 12 -14 mm and diameter 8 – 10 mm, pedicel length very small 1 - 1.5 mm, calyx cup shaped and diameter 2 - 2.42 mm, number of sepals usually 5 but rarely 4, petals usually 5 but sometimes recorded 3 or 4 petals oval shaped and size 7 - 7.2 mm X 4.2 - 6mm. The flower was protogynous and the stamens were longer than pistil. The pistil was small and stud shaped and length was 3.12 mm. Style was very short and fixed in the cavity of ovary and length around 1 mm. Ovary length was 1.16 mm and diameter 2.33 mm and locules per ovary 14. The stamens were free and numbers 24 - 29, stamen average length was 6.05 mm (filament 4 and anther length 2 mm). Stigma was capitates and length and diameter 1.41 mm ×1.68 mm. Length

of stamens were longer than pistil and 30 - 40% rudimentary pistils were recorded. Pollen shape was usually rounded and diameter 25.44 µm. Percentage of viable pollens was 75.08% and germination 34.97%.

The fruit shape was found usually obloid but frequently spheroid, shape of the fruit base was truncate and the apex rounded and depressed. The surface of rind color were light yellow, green yellow and light green.. The albedo part was white, smoothness of rind surface "smooth & pitted", bitterness was tested organoleptically and recorded nil, low, medium and high. The pulp color was creamy white and taste highly sour, juice color was creamy white. seed shape semi deltoid and color was creamy and fruits harvesting month was October. The fruit weight was 165 - 260g per fruit, size 4.5 cm – 6 cm X 5.2 -7.25 cm, peel thickness 8.20 mm, number of segments 14 and juice content 131 ml per fruit. The days required from fruit set to harvest 175.55, fruits per plant was recorded 12.20 and seeds per fruit was 6 -11.

Flower drop were 92.81% in 2009, 98.32% in 2011.Initial fruit set percentage after 21 days were 7.39% in 2009 and 01.56% in 2011. Final fruit set after 120 days were 0.88% in 2009 and 0.24% in 2011.

The floral bud development was divided into six stages At the stage **I,** purple color bud primordial were seen and the length was around 1mm at 7 days old and diameter was greater (2.25 mm). After 14 days at the stage of **II,** the buds were at tiny stage (4.70 mm **X** 5.11 mm), pedicel and calyx were visible and hairy with green color. After 21 daysat the stage **III**, the bud became whitish but at the top purple cross margin remained and size increased rapidly at this stage (8.21 mm **X** 9 mm) which almost double than previous stage. At the stage **IV** (28 days) size slightly increased and in next stage (**V**) size almost same (10.1 mm and 11.9 mm) respectively. After 35 days the bud reached at maximum size. The dehiscence of pollens and stigma became receptive at the stage **V**, pollination, fertilization were done before the anthesis. After this stage the flower has started to open and fertilized ovary (fruit) shown with yellow stigma, white petals and yellowish stamens.

The total growth pattern of fruits were divided into seven stages. At the stage **I,** after 10 -1 2 days from initial fruit set (40 days from bud initiation), generally the light yellowish green ovary with yellow stigma was attached. Most of the fruits dropped at this stage attaining length 0.39 cm and diameter 0.27 cm. From stage **I** to stage **II**

the increament of length and diameter of fruit were very rapid. At stage **II,** the length and diameter were 1.35 cm and 2.2 cm respectively.The length and diameter of fruit from **III** to **VII** were, at stage **III** 1.85 × 3.10 cm, at stage **IV** 2.35 × 3.90cm, at stage **V** 3.25 × 4.85 cm, at stage **VI** 4.70 × 6.20cm and at stage **VII** 4.71 × 6.52 cm respectively.

The color of the fruit remain deep green at the stage **II** and **III,** green at stage **IV** and **V**. At the stage **VI** the color of the fruit start to light green and yellowish green at stage **VII** .After harvesting or after stage **VII** the rind color of fruit became yellow. Fruit attained the harvesting maturity in 200 - 230 days from flower bud inititiation. The rate of increase of fruit was slower during June and rapid increase took place from mid July to mid August.

Among the ten germplasm studied, the CM -001 was found superior in respect of floral and fruit characteristics.

The mitotic chromosomes of Satkara root cells were prepared by Orcein staining method. All slides showed 2n = 18 without any numerical variations. Individual chromosomes length ranged from 0.05 to 1.89 µm. The total length of 2n complement is 24.30 µm. The relative length of each chromosome was ranging from0.04 to 0.08. No satellite or secondary constriction was found.

The CMA banding of root cell's chromosome was determined by fluorescent method. GC rich many CMA-banded dot like yellowish positive bands (10-15) in mitotic inter-phase and prophase and 8 CMA-positive bands in mitotic metaphase. Total length of CMA - positive banded region was 4.45 µm which covered 18.30 % of the total chromatin length.

PART - II

PHYTOCHEMISTRY

Chapter - 5

Materials and Methods

PHYTOCHEMISTRY

Materials and Methods

Materials

Fresh fruits of *Citrus macroptera* Mont.Var.*annamensis* were collected from the Citrus Research Station, Bangladesh Agricultural Research Institute (BARI), Jaintapur, Sylhet. A voucher specimen was submitted to the National Herbarium of Bangladesh, Mirpur, Dhaka and identified with the accession number – DACB 34217. Brine shrimp eggs and microrganisms

Equipments:

Porcelain crucible, muffle furnace, desiccator, Balance, long necked digesion flask (Kjeldhal flask), 100 ml volume tire flask, round bottom 500 ml flask, Soxhlet apparatus, fine (muslin) cloth, beakers of different volume, pH meter (*Model* : Jenway instrument), refractometer (Model. Abbe), hand pastle, conical flask of different volume, test tubes, spectrophotometer (UV - 1201, UV - VIS, Shimadzu, Japan), Flame emission spectropho -tometer (*Model:* Jenway, PEP7, Jackson, 1967). Atomic Absorption Spectometer (AAS), Innowax, FSC column (60 m ×0.25 mm, 0.25 μm film thickness), TLC plate (0.25 mm thickness), filter paper discs, petridishes, inoculating loop , sterile cotton, sterile forceps, spirit burner, micropipette , screw cap test tubes , masks and gloves, laminar air flow hood , autoclave ,Incubator, refrigerator, lamp to attract shrimps, pipettes, micropipette, glass vials, magnifying glass, test tubes, small tank with perforated dividing dam to hatch the shrimp.

Reagents:

Conc. H_2SO_4 (BDH, England), Na_2CO_3 solution (BDH), NaOH, (BDH), digestion mixture (BDH), methyl orange – indicator(BDH), pumice stones (E.Merch,Germany), petroleum ethelene (Merck), extraction thimble , ethyl alcohol (Merck) , ether (Merck), buffers solution (BDH) , 5% metaphospheric acid in 10% acetic acid solution (BDH), 2,4 – dinitro - phenyl hydrazinc (BDH), Acid washed norit (black charcoal powder (BDH), Standard ascorbic acid solution (Sigma Chemical Co, USA), Conc. HCl (Merck), Phenlopthelin indicator (Merck) , ammonium sulphomolybdic acid solution (BDH), 2,4 - dinitrophenol indicator (BDH), Freshly prepared stannous chloride solution (BDH) , EDTA solution (BDH), Potassium ferocyanide (Merck), Hydroxylamine hydrochloride (Merck), Tri - ethamolamine (TEA), Eriochrome Black-T indicator (BDH), *n* -hexane (Merck), 2,2–diphenyl-1-picryl hydrazyl (DPPH), Methyl alcohol (Merck), Silica gel (Merck), Trolox^R (Sigma - Aldrich, UK), Resazurin (BDH), Ciprofloxacin (purchased from Fluka Chemie AG, Bucks), Ferric chloride (Merck) , Olive oil (BDH), Ammonium hydroxide (BDH), Chloroform (Merck), Dimethylsulphoxide (DMSO) (BDH) , Vincristine sulphate (BDH) , NaCl salt (BDH) , Nutrient agar (BDH).

Methods

A) Proximate composition, crude fiber and energy (Kcal)

1. Total Ash

To determine total ash 5 g of the fresh peel was taken on a porcelain crucible which was previously heated to 100^{0}C and cooled and weighed (Joslyn,1950). The crucible was heated in a heater till all material is completely charred, then heated in a muffle furnace for about 5 hrs at 600^{0}C. It was then cooled in a desiccator and weighed. To ensure completion of ashing the crucible was again heated muffle furnace for half an hr, cooled and weighed again. The three weights were taken following such ways and average was recorded.

Calculation =

$$\text{Ash content (g/100g)} = \frac{\text{Wt. of the ash X 100}}{\text{Wt. of the sample}}$$

2. Moisture content

Moisture was estimated according to the method of Pearson, (1970).This involve the measurement of the weight lost due to evaporation of water. Peel sample(5mg) was taken in a porcelain crucible which was previously heated 100^{0}C and cooled in desiccator followed by weighing. The crucible was then placed in an oven and heated about 4 hrs at 100^{0}C. It was then cooled at room temperature in desiccator and weighed. The process of heating, cooling and weighing were repeated to the constant weight.

Calculation=

$$\text{Moisture content} = \frac{\text{Wt of the moisture X 100}}{\text{Wt. of the sample}}$$

3. Protein content

The protein content of the sample was determined by semi-micro Kjeldhal method as described by AOAC (AOAC,1984).

Digestion

Peel sample (2 g) was weighed and dropped into a 500 ml Kjeldhal flask. To this 20 ml of conc.H_2SO_4 solution and 5gm of digestion mixture were added. The flask was then heated carefully at a low temperature until the mixture no longer froths, and then the temperature was increased so that the flask boiled briskly. The flask was occasionally rotated and agitated to ensure the complete digestion of samples. The mixture was digested until the black carbon deposit was oxidized and the solution became clear. To ensure complete digestion the heating was continued for an additional hrs. The flask was then cooled and digested materials were transferred into a 100 ml volume flask and distilled water was added to make it 100 ml. A blank was run simultaneously with all the reagents except sample.

Distillation

The distillation apparatus was thoroughly washed with distilled water before the experiment. 10 ml of 0.IN sulphuric acid containing two drops of indicator was taken into the receiving beaker and it was so placed that the lower part of the delivery tube extending from the condenser was immersed into it. A few pieces of pumice stones were added into a round bottom 500 ml flask to prevent the bumping. Fifty ml of digested solution was taken to the flask, and then sufficient amount of 40% sodium hydroxide (30 ml) was added to make the content alkaline. The flask was immediately connected with the distillation system. The solution was first heated slowly and then the heating was so adjusted that the mixture boiled such a rate that water and ammonia distilled over a moderate rate. The distillation was continued until the volume of the receiving flask became approximately doubled. At the end of distillation, the distilling condenser was then rinsed with water and the washing was added to the distillate. The ammonia absorbed in the receiving flask was titrated back with 0.IN NaOH. The three titration data were taken.The reagent blank was distilled

and titrated similarly. The percentage of crude protein was calculated from the formula given below.

Calculation = Protein content (g/ 100 g sample) = (c - b) X 1.4d X 6.25 X 100 / a

Where
a = weight of the sample (g)
b = volume of alkali for sample titration (ml)
c = volume of alkali for blank titration (ml)
d = strength of alkali (normality N)
6.25 is gravimetric factor for proteins in foods (15% N in protein).

4. Fat content

The Soxhlet method was used to determine the lipd content of the material by (AOAC, 1990). The fat was extracted from the dried sample (5 g) using petroleum ether as a solvent. The sample was taken into a pre dried extraction thimble with porosity permitting a rapid flow of petroleum ether. 100 ml of petroleum ether was taken into a boiling flask. Soxhlet extractor was extracted at a rate of 5 to 6 drops per second condensation. Each extraction continued eight hrs. At the end the flask containing oil was cooled and weighed. Three data were taken and average was recorded.

Calculation =

$$\text{Lipid content (g/100 g)} = \frac{\text{Wt. of the extract X 100}}{\text{Wt. of the sample taken}}$$

4. Carbohydrate content

The content of carbohydrates was obtained by differences i.e., by subtracting the sum of average values of ash, moisture, protein, crude fat and crude fiber from the 100 g of dry sample (ICOMR,1976)

Calculation = Carbohydrate (g %) = 100 – (moisture + protein +fat + ash) g/100 g

6. Crude fiber

Crude fiber is the organic residues and consists largely of cellulose together with little lignin (ICOMR, 1976).

Procedure

Peel paste (5 g) was taken into 500 ml beaker and 200 ml of boiling 0.255N H_2SO_4 was added. The mixture was then boiled for 30 minutes keeping the volume constant by adding water on frequent intervals. At the end of this period the mixture was filtered through a fine (muslin) cloth and the residue was washed with hot water till free from acid. The material was transferred to the same beaker and 200 ml of boiling 0.31N NaOH was added. After boiling for 30 minutes (keeping the volume constant as before) the mixture was filtered through cloth. The residue was washed with hot water until free from alkali followed by washing with some ethanol and ether. It was then transferred to crucible and weighed and to dry at 80 - 100°C for 3 hrs. Cooled and weighed again. Three data were taken and average was recorded. The difference of the weight represented the weight of the crude fiber. The percentage of crude fiber was calculated by the following formula given below.

Calculation =

$$\text{Crude fiber content (g / 100 g of the sample)} = \frac{\{100 - (\text{moisture} + \text{ash})\} \times \text{weight of crude fiber}}{\text{Wt. of the sample}}$$

7. Energy

Total energy from fresh peel (sample) was calculated by multiplying 4 with carbohydrate, 9 with total fat and 4 with total protein value. From the summation of these values the total calorie was estimated (Osborne and Voogt, 1978)

B) Estimation of pH, TSS, Ascorbic acid and Organic acid values

1. The pH

Fresh juice (10 ml) or fresh peel (10 g) extract sample were taken into a beaker and added 50 ml distilled water and stirred well. Then switched on the pH meter (*Model*: Jenway instrument, pH meter 3305, Jackson, 1967) and adjusted with buffers. Placed the electrodes into the sample solution and taken reading three times.

2. Total soluble solid (TSS)

TSS content of the fruit juice and peel extract was estimated by using hand Refractometer (Model. Abbe). A drop of sample was placed on the prism of the Refractometer. Percent of TSS was obtained from direct reading from the instrument. Temperature was corrected (Rangana, 1979).

3. Estimation of ascorbic acid by spectophotometric method (Roc and Asterlin, 1944).

From freshly cut fruit, 1 g of peel and 1 g of juice were measured seperately. Incase of pee it was homogenized in a mortar with a pastle using 10 ml of 5% metaphospheric acid in 10% acetic acid solution and the mixture was then

filtered by suction. In case of juice 5% metaphospheric acid in 10% acetic acid solution was added and the mixture was then filtered by suction.

The extract was collected in a conical flask and 1gm acid washed norit (black charcoal poder) was added to it and shaken vigorously for 10 minutes. The mixture was filtered by sintered glass filter. The solution gave whitish color. Sample thus prepared was made 10 ml with the extraction solution and kept overnight in a refrigerator. 1 ml of norit filtrate, 1 ml of working standard (all of the different concentration) and 1 ml of metaphospheric acid (as blank) were taken in different test tubes. Then 0.25 ml of 2,4 - dinitro-phenyl hydrazine was added to each of the test tubes containing sample extract, standard ascorbic acid and the blank solution. The tubes were then placed in a water bath at 60°C for 1 hr. After the incubation, the tubes were removed and placed in ice bath, 1.25 ml of 85% sulphuric acid solution was added drop wise slowly and carefully to each test tube (throughout the analysis the timing is very important to maintain). All the tubes were vortexed carefully and left at room temperature for 30 minutes.

The absorbance was measured at 520 nm in a spectrophotometer (UV - 1201, UV -VIS, Shimadzu, Japan).The reading was taken against the sample blank. The ascorbic acid was calculated by using the following formula.

Calculation =

$$\text{Ascorbic acid mg/100 g of sample} = \frac{\text{Sample absorbance X volume of stock X volume of standard factor} \times 100}{\text{Standard absorbance X Sample wt (g)}}$$

4. Citric acid and Malic acid

Juice & peel extracts of were collected & added 10 ml of 0.01M NaOH to juice & peel separately. Then stirred the each mixture continuously and added 50 ml of hot distilled water & again stirred it. After cooling the mixture, it was back titrated against 0.01M HCl using Phenlopthelin indicator. At the end point, purple color was observed. The results are expressed in mg /100 g.

C) Estimation of mineral nutrients

Sodium and potassium

Sodium and potassium of both peels and juice were determined by flame emission spectrophotometry (*Model:* Jenway, PEP7, Jackson, 1967).

1. Sodium

At first prepared standard sodium solution by dissolving 2.542 g of NaCl crystals in water, diluted the solution to 1000 ml and mixed it. This solution contains 1000 ppm Na. Read the Na on a flame photometer (at 589 & 589.6 nm) by comparing the emission with that from standards made in distilled water containing 0, 5, 10, 20 and 40 ppm of Na. The results obtained from the photometer reading are compared with various standards in the graphs and expressed in ppm.

2. Potassium

At first prepared standard potassium solution by dissolving 0.9527 g of dried KCl in water, diluted the solution to 500 ml and mixed it. This solution contains 1000 ppm K. Read the K on a flame photometer (at 766 & 769 nm)by comparing the emission with that from standards made in distilled water containing 0, 5, 10, 20 and 40 ppm of K. The results obtained from the photometer reading were compared with various standards in the graphs and expressed in ppm.

3. Phosphorus

Phosphorus was evaluated by "Molebdophosphoric blue color method in Sulphuric acid system." Peel and juice sample (5 g each) was taken in a 250 ml beaker and added 2 ml of ammonium sulphomolybdate solution and mixed thoroughly. Then added 2 drops of 2,4 -dinitrophenol indicator to the solution and it gave yellow color, and added N/20 HCl drop wise until it became colorless. The colorless solution

indicated that pH below 3, again added N/20 NaOH drop wise just until yellow color appears and finally added N/20 HCl until yellow became faint. Added 3 drops of freshly prepared stannous chloride solution and developed blue color and made the volume up to the mark and shacked again thoroughly.Transferred 10 ml of clear solution to spectophotometer tube/cell and read the intensity of the color in a spectophotometer at 660 nm.

Preparation of standard solutions for standard curve

Dissolved 0.2195 g of potassium di -hydrogen phosphate in about 400 ml distilled water in a 1000 volumetric flask, and added 25 ml of 7N H_2SO_4, mixed and diluted to the mark with water, giving 50 ppm of P. For 0.5 standard, diluted 5 ml of 50 ppm stock solution in 500 ml. Diluted 5 ml, 10 ml, 15 ml and 20 ml of 0.5 ppm solution in 50 ml volumetric flasks to obtained 0.05, 0.10, 0.15, and 0.20 ppm concentration respectively.

Calculation =

$$\text{Amount of P in ppm} = \frac{\text{ppm from the standard curve} \times 50\text{ml volume of extraction} \times 50\text{ ml dilution}}{\text{Amount of sample (g)} \times \text{volume of extract used}}$$

Calcium and Magnesium

Calcium and Magnesium in citrus juice and peel were determined by complexometric titration method.

3. Calcium

Sample (5 ml) solution was taken in a small porcelin cup. Added 5 ml of 10% NaOH to the solution and finally added 0.02 g Murexic indicator and titrated against 0.01N EDTA solution. At the end point the color was changed from pink to light blue. Standerized 0.01N EDTA solution with the help of standard Ca solution using all the

reagents used in the above titraion.The methods of titraion used 3 times and average was made

Calculation =

1000 ml of 1N EDTA = 20 g of Ca

1ml of 1N EDTA = 0.02 g of Ca

$$Ca^{+}\text{in ppm} = \frac{0.02 \times \text{ml of EDTA used} \times \text{normality of EDTA} \times 100 \times 1000}{\text{ml of sample (aliquot) used}}$$

5. Magnesium

Sample solution (5ml) was taken in a small porcelain cup. Added 25ml of distilled water, 15 ml of buffer solution, 10 drops of potassium ferocyanide.10 drops of hydroxylamine hydrchloride, 5 drops of Tri-ethamolamine (TEA).

When all the reagents have been added, warm the solution gently for 2-3 minutes, cooled and added 3 drops of Eriochrome Black -T indicator. Standarized 0.01N EDTA solution with the help of standard Mg solution using all the reagents used in the above titration.The methods of titration used 3 times for accurate results.

Calculation =

1000 ml of 1N EDTA = 12 g of Mg

1 ml of 1N EDTA = 0.012 g of Mg

$$\text{Mg in ppm} = \frac{0.012 \times \text{ml of EDTA used} \times \text{normality of EDTA} \times 100 \times 1000}{\text{ml of sample (aliquot) used}}$$

D) Antimicrobial activity of peel extract

Antimicrobial activity was evaluated by disc diffusion method (Bayer *et.al.*, 1966). It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials (Ronald, 1982). The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institution of Nutrition and Food Sciences (INFS), University of Dhaka. Both gram positive, gram negative and fungi were taken for the test and they are listed in Table 2.1.

1. Test organisms

Table : 2.1 List of bacteria and fungi used in disc diffusion method.

Gram positive bacteria	Gram negative bacteria	Fungi
Bacillus cereus	*Escheritia coli*	*Candida albicans*
Bacillus megaterium	*Pseudomonus aeruginosa*	*Aspergillus niger*
Bacillus subtilis	*Salmonella typhi*	*Sacharomyces cerevacae*
Staphylococcus aureus	*Salmonella paratyphi*	
Sarcinia lutea	*Shigella boydii*	
	Shigella dysenteriae	
	Vibrio mimicus	
	Vibrio parahemolyticus	

2. Nutrient culture medium and their composition.

Nutrient agar medium

Ingredients	Amounts
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 25^0C

3. Preparation of culture medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The content was heated in a water bath to make a clear solution. The pH (at 25^Oc) was adjusted at 7.2 to 7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15 lbs pressure at 121^0C for 20 minutes. The slants were used to making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

4. Sterilization procedures

In order to avoid any type of contamination and cross contamination of the test organisms the antimicrobial screening was done under laminar airflow hood and all types of precautions were maintained. UV light was switched on one hr before working. Petridishes and other glassware were sterilized by autoclaving at a temperature 121^0C and a pressure of 15lbs/sq inch for 20 minutes. Micropipette, blades , cotton, forceps, blank discs etc. were also sterilized.

5. Preparation of subculture

In an aseptic condition under Laminar air flow cabinet, the test organisms were transferred from the pure culture to the agar slants with the help of transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hrs at 37^0C for their optimum growth. These fresh cultures were used for sensitivity test.

6. Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspensions were immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distributions of the test organisms in the media.

7. Preparation of discs

Three types of discs were used for antimicrobial screening –

a) Standard discs: These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation Ciprafloxacin (30 µg/disc) standard disc was used as the reference.

b) Blank discs: These were used as negative controls which ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

c) Preparation of sample discs: The amount of ethanolic peel extract was dissolved in specific volume of solvent (Chloroform) to obtain the desired concentrations in an

aseptic condition (300 µg/ disc). Sterilized filter paper discs were taken in a blank petridish under the laminar airflow hood. Then discs were soaked with the solutions of test samples and dried, so that no residual solvent is there.

7. Diffusion and incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with the test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hrs upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in a incubator at 37^0 C for 24 hrs.

9. Determination of antimicrobial activity by measuring the zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with Vernier scale.

E) Brine shrimp lethality bioassay of peel extracts

The bioassay was carried out according to the principle and protocol described by Meyer *et al.,* (1982), McLaughlin *et al.,* (1991) with slight modifications. Brine shrimp eggs (*Artemia salina*) were placed on one side of a small tank which was filled with boiled, filtered sea water, covered with aluminum foil, and fully aerated. After 48 hrs incubation at room temperature and under illumination, the resulting nauplii (larvae) were attracted to the other side of the tank with a light source and collected with a Pasteur pipette. Samples for testing were prepared by initially dissolving 50 mg of crude extract in 5 ml of dimethyl sulfoxide (DMSO) and further

diluted with sea water to produce the required concentrations. Appropriate amounts (500 , 50 , or 5 µL for 1000, 100, and 10 µg/ml, respectively) were transferred to vials containing small filter paper discs, air-dried overnight to evaporate the solvent, and further dried under nitrogen gas. Ten brine shrimps were transferred to each sample vial and boiled filtered sea water was added to made 5 ml. Tests for each concentration were done in triplicate. A control experiment containing 500 , 50 , or 5 µL DMSO in 5 ml of boiled, filtered sea

water and ten brine shrimps was also performed in triplicate for each concentration. The vials were maintained under illumination. Survivors were counted after 24 hrs and the percentage of mortality at each vial and control was determined using the equation =

% of mortality = (No. of dead nauplii/ initial no. of live nauplli) x 100

A formal analysis by Finney (1971) was used to determine the concentration at which lethality to brine shrimp represents 50% (LC_{50}). LC_{50} values less than 100 ppm (or 100 µg/mL) were considered significant (Gupta, 1996).

E) Composition of the volatiles (essential oil)

1. Volatile oil extraction

Fresh peels (flavedo and albedo, 100 g) from the fruits were cut into small pieces by clean sharp knife. The peels were placed in a 1L round bottom flask. Distilled water (600 ml) was added to facilitate distillation process. The flask was fitted with distillation apparatus. The distillate was collected in a flask. The distillation was continued about 4 hrs till to oily drops appear. The distilled portion was extracted 3 times with 50 ml *n* -hexane immediately after distillation. The *n* -hexane part was

dried over anhydrous sodium sulphate. The extract was evaporated to dryness in a vacuum evaporator to obtain the volatile oil.

2. **Gas Chromatography – Mass spectrometry (GC- MS) analysis**

The GC - MS analysis was performed in an Agilent 5975 GC - MCD system. Innowax FSC column (60 m ×0.25 mm, 0.25 µm film thickness) was used with helium as a carrier gas (0.8 ml/min). GC oven temperature was kept at 60^{0}C for 10 minute and programmed to rise 220^{0}C at a rate of 4^{0}C/min, and kept constant at 220^{0}C for 10 minute and then programmed to rise to 240^{0}C at a rate of 1^{0}C/min. Split ratio was calibrated at 40:1. The injector temperature was set at 250^{0}C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

3. **Gas Chromatography (GC) analysis**

The GC analysis carried out using an Agilent 6890N GC system. The FID detector temperature was 300^{0}C. To obtain the same elusion order as with GC - MS, simultaneous auto injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated thrice from FID chromatograms. The results are presented as ± standard deviation (SD) (n = 3). The experiment was done in three replicates.

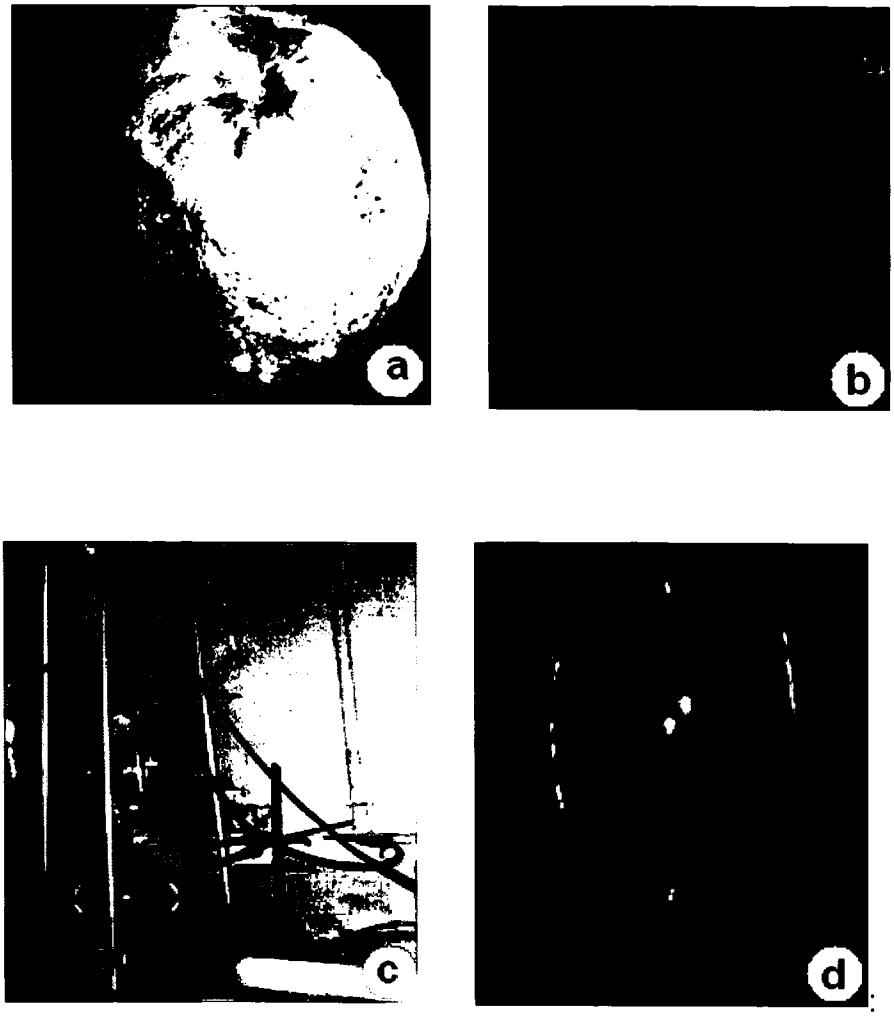

Plate: 2.1 Soxhlet apparatus and extraction of essential oils (a) mature fruit (b) slices of peel (c) Soxhlet apparatus (d) oil mixed with ethanol.

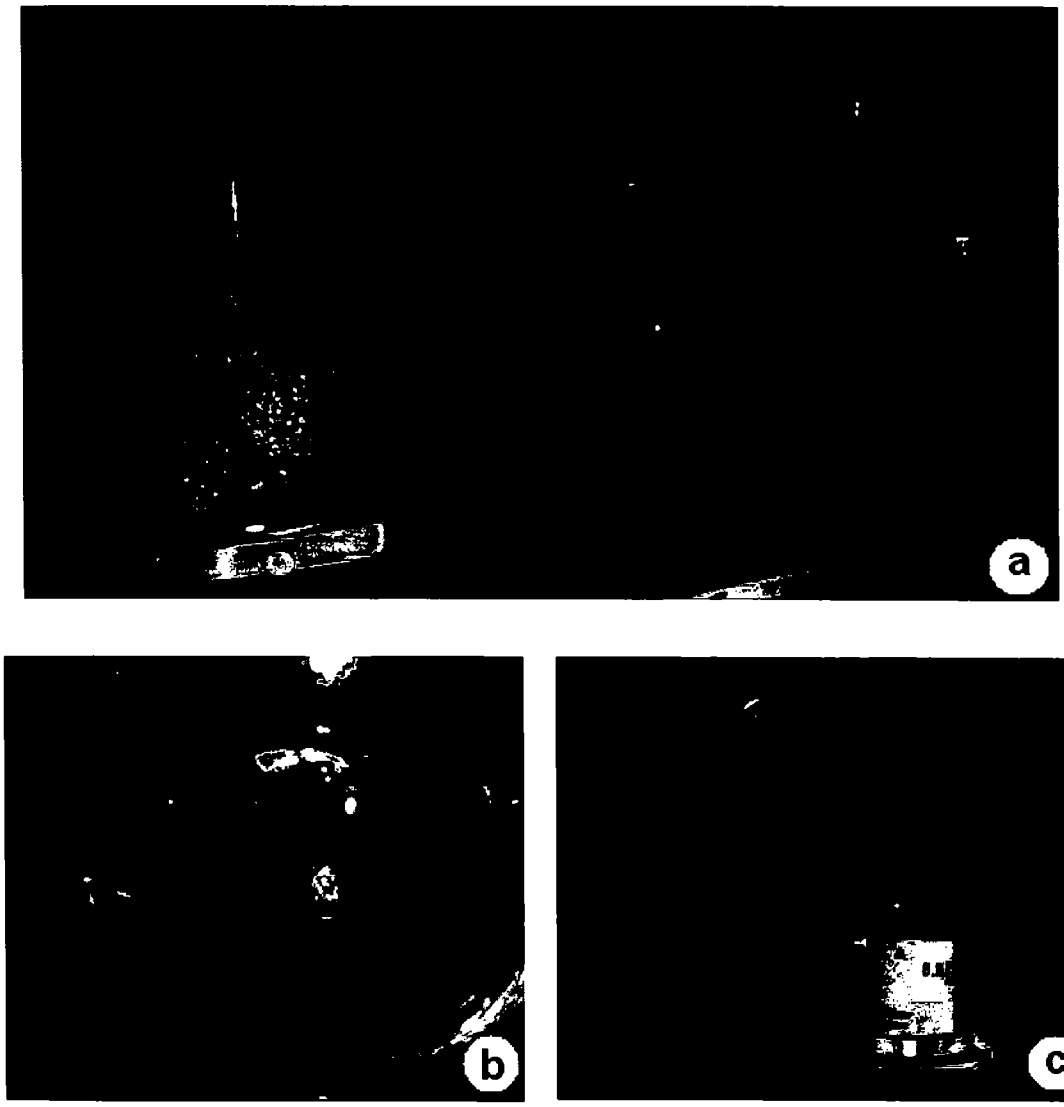

Plate: 2.2 Steam distillation plant (a) Steam distillation apparatus (b) collection of oil mixed with water (c) evaporotator machine.

4. Identification of components

Identification of the volatile oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n* - alkanes. Identification of compounds was based on computer matching using the Wiley GC - MS Library, Adams library and mass finder 3 Library (McLafferty and Stauffer,1989. Koenig *et .al.*, 2004) and the MS data was compared with literature data (Joulain and Koenig, 2004. ESO 2000).

F) Bioactiviies of volatile oil

1. Free radical scavenging activity (Qualitative 2, 2- diphenyl -1-picryl hydrazyl (DPPH) assay)

DPPH, molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG Bucks. The free radical-scavenging effect of the volatile oil (5 mg/ml) was assessed using the qualitative DPPH (Kumarasamy *et al.,* 2002 and Kumarasamy *et al.,* 2007). DPPH (8 mg) was dissolved in Methyl alcohol (100 ml) to obtain a concentration of 80 mg/ml.Test samples were applied on Silica gel precoated TLC plate (0.25 mm thickness), air dried and sprayed with DPPH solution using an atomizer. It was allowed to develop for 30 minutes. The color changes (purple on white) Trolox^R (1 mg/ml), purchaged from Sigma-Aldrich, UK, was used as positive control.

2. Antibacterial assay incorporating resazurin as an indicator of cell growth

The antibacterial activity of volatile oil was assessed against six bacterial strains,*Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (NCTC 10400), *Escherichia coli* (ATCC 8739), Amphicilin resistant *Escherichia coli (*NCTC 10418), *Staphylococcus aureus* (NCTC 1803) and *Salmonella typhii* (NCTC 10203). The recently published 96 - well microtitre assay using Resazurin as the indicator of cell growth (Sarker *et al.,* 2007) was employed for the determination of the minimum inhibitory concentration (MIC) of the volatile oil. Ciprofloxacin (purchased from Fluka Chemie AG, Bucks), a broad spectrum antibiotic, was used as positive control. The bacteriostatic/ bactericidal property of the volatile oil was carried out as follows: agar plate was seeded with the mixture from the well

which was just before the well of the MIC, using sterile loop and kept at 4^0C to facilitate maximum diffusion. The plates were kept in an incubator (37^0C) to allow the growth of the bacteria. Any bacterial growth would indicate the bacteriostatic property of the extract, and no growth would be an indicator of bactericidal activity.

G) Qualitative test of some phytochemical constituents

Phytochemical screening

Peels from fresh fruits were blended by blender machine. 100 g of materials soaking in 200 ml distilled water for 12 hrs. The extracts were filtered using Whatman paper No 42. Chemical tests were carried on the aqueous extract using the standard procedures to identify the constituents as described by (Sofowara, 1993), Trease and Evans, (1989) and Harborne, (1973).

1. Test for tannins

The sample extract (2 ml) was boiled in 20 ml water in a test tube and then filtered. Few drops of 0.1% ferric chloride was added and observed the formation of coloration.

2. Test for saponins

About 2 ml of sample extract was boiled in 20 ml of distilled water in water bath and filtered. 10 ml of filtrate was mixed with 5ml of distilled water and shaken vigorously for stable persistent forth. The forthing was mixed with 3 drops of olive oil and shaken vigorously and observed.

3. Test for alkaloids

Fresh sample (2 g) was weighed into a 100 ml beaker and 50 ml of 10% acetic acid in ethanol was added and covered and allowed to stand 4 hr. This was filtered and the extract was concentrated on a water bath to one quarter of the

original volume. Concentrated ammonium hydroxide was added drop wise to the extract till to positive indication.

4. Test for flavonoids

Dilute ammonia solution (5 ml) was added to a portion of the sample extract was followed by addition of concentrated sulphuric acids and observe the formation of yellow coloration.

5. Test for steroids

Acetic anhydride (2 ml) was added to 1gm of ethanolic extract of the sample with 2 ml of H_2SO_4 .The color change of the sample was observed,

6. Test for terpinoids (Salkowki's test)

A sample extract (5 ml) was mixed with 2 ml of chloroform, 3 ml of concentrated H_2SO_4 was added to form a color layer. A reddish brown coloration of interface indicated the presence of terpinoids.

7. Test for cardiac glycoside (Killar- Killani test)

A sample extract (5 ml) was mixed with 2 ml glacial acetic acid containing one drop of ferric chloride solution, then added 1ml of concentrated H_2SO_4. The formulation of raddish brownish ring at the interface will indicated the presence of cardiac glycoside.

Chapter - 6

Results

Results

A) Proximate composition, crude fiber and energy (Kcal)

The ash, moisture, protein, carbohydrate, fat, dietary fiber and energy values are presented in Table 2.2. The ash percentage of the peel was analyzed and found the value 0.89 %. The moisture content of the peel also counted 75.53%. Protein was analyzed through semi-micro Kjeldhal method and was recorded 1.02 g for peel and 0.75 g for juice. Carbohydrate for peel was recorded 18.98 g. Fat counted for peel and juice was 0.85 g and 0.0.43 g respectively. Crude fiber for peel 2.46% and 1.54% for juice were recorded. Energy was calculated 90 Kcal for peel and 83.23 Kcal for juice.

Table: 2.2 Proximate composition, crude fiber and energy (Kcal) of Satkara fruit.

Name of analysis	Material (peel)	Juice
Ash(%)	0.89 ± 0.01	---
Moisture (%)	75.53 ± 0.06	_
Protein (g/100g)	1.02 ± 0.02	0.75 ± 0.03
Carbohydrate (g/100g)	18.98 ± 0.32	_
Fat (g/100g)	0.85 ± 0.08	0.43 ± 0.04
Crude fiber (%)	2.46 ± 0.24	1.54 ± 0.03
Energy (Kcal/100g)	90 ± 0.56	83.23 ± 0.43

B) The pH, TSS, ascorbic acid and organic acid values of satkara.

The pH, TSS, ascorbic acid (Vit-C) and organic acid values of Satkara are presented in Table 2.3.

Table: 2.3 The pH, TSS, ascorbic acid and organic acid values of Satkara fruit.

Name of analysis	Material (peel)	Juice
pH	4.32 ± 0.13	2.45 ± 0.04
TSS (%)	24.47 ±0.13	12.43 ±0.21
Ascorbic acid (mg/100g)	5.58 ± 0.77	53.07 ± 0.69
Citric acid (mg/100g)	4.53 ±0.32	55.25 ±0.32
Malic acid (mg/100g)	1.43 ±0.05	4.54 ±0.21

The pH value of peel extract was 4.32 and 2.45 for juice. Total soluble solid (TSS) of peel was recorded 24.47% whereas juice contained 12.43%. Ascorbic acid (Vit-C) of peel was poor (5.58 mg) but high in juice (53.07 mg) per 100 g. Citric acid is an important organic acid and obtained 04.53 mg through peel analysis and 55.25 mg from juice respectively. The malic acid was recorded 01.43 mg for peel and 04.54 mg for juice.

C) Mineral components

The results of nutrient analysis are presented in Table 2.4 .In present study Sodium and potassium were higher, Na = 3.58% for peel and 2.57% for juice, K = 2.14% for peel and 1.14% for juice. Next to Na & K, Magnesium, Phosphate, calcium were

recorded 0.38% ,0.20%, 0.19% for peel. In case of juice, these values were 0.14%, 0.07 % and 0.21% respectively.

The trace components (Fe, Mn, Zn & Cu) of the fruit peel and juice are also presented in the Table 2.4 .The values of Fe, Mn, Zn & Cu for peel were 0.33%, 0.13%, 0.07%, and 0.06% for peel and 0.31%, 0.17%, 0.26%, and 0.05% for juice respectively.

Table 2.4 Mineral composition of peel and juice of Satkara) (Dry weight basis).

Name of analysis	Material (peel)	Juice
Sodium (%)	3.58 ± 0.15	2.57 ± 0.13
Potassium (%)	2.14 ± 0.05	1.14 ± 0.12
Magnesium (%)	0.38 ± 0.04	0.14 ± 0.01
Phosphate (%)	0.20 ± 0.01	0.07 ± 0.00
Calcium (%)	0.19 ± 0.03	0.21 ± 0.03
Iron (%)	0.33 ± 0.06	0.31 ± 0.03
Manganese (%)	0.13 ± 0.02	0.17 ± 0.02
Zinc (%)	0.07 ± 0.01	0.26 ± 0.03
Cupper (%)	0.06 ± 0.00	0.05 ± 0.00

D) Antimicrobial activity

The results of antimicrobial activity of ethanolic peel extracts of Satkara are presented in Table 2.5.

Table: 2.5 The inhibition zone of test samples and a standard antibiotic.

Test microbes	Ciprofloxacin (zones)	zone of inhibition
Gram positive bacteria		
Bacillus cereus	52	-
Bacillus megaterium	56	-
Bacillus subtilis	54	-
Staphylococcus aureus	50	09
Sarcina lutea	54	
Gram negative bacteria		
Escherichia coli	51	08
Pseudomonus aureus	53	
Salmonella typhi	55	
Salmonella paratyphi	54	
Shygella dysenteriae	54	
Vibrio parahaemolyticus	55	
Vibrio mimicus	53	08
Shigella boydii	50	08
Fungi		
Aspergillus niger	51	08
Candida albicans	51	09
Saccharomyces cerevaceae	50	08

The antimicrobial activities of the peel extracts of Satkara were observed in the present study. The zones of inhibition produced by the crude ethanolic extracts were ranged of 8 - 9 mm at a concentration of 30 µg/disc. None of the Gram positive bacteria except *Staphylococcus aureus* showed any zone of inhibition. Whereas in case of Gram negative bacteria only *E.coli, Vibri mimicus* and *Shigella boydii showed* 8 mm zone of inhibition. All the fungi showed 8 - 9 mm zone of inhibition.

E) Brine shrimp lethality assay

The lethality of the extracts to brine shrimp was determined and the results are given in Table 2.6 and 2.7. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples. The ethanolic extract of the peels (ECM) of *Citrus macroptera* were subjected to brine shrimp lethality bioassay following the procedure of Meyer *et al.,* (1982).The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Vincristine Sulfate (VS) was used as positive control and the LC_{50} was found 0.451 µg/ml. Compared with the negative control (sea water), VS gave significant mortality. The LC_{50} values of ECM was found 2.53 µg/ml.

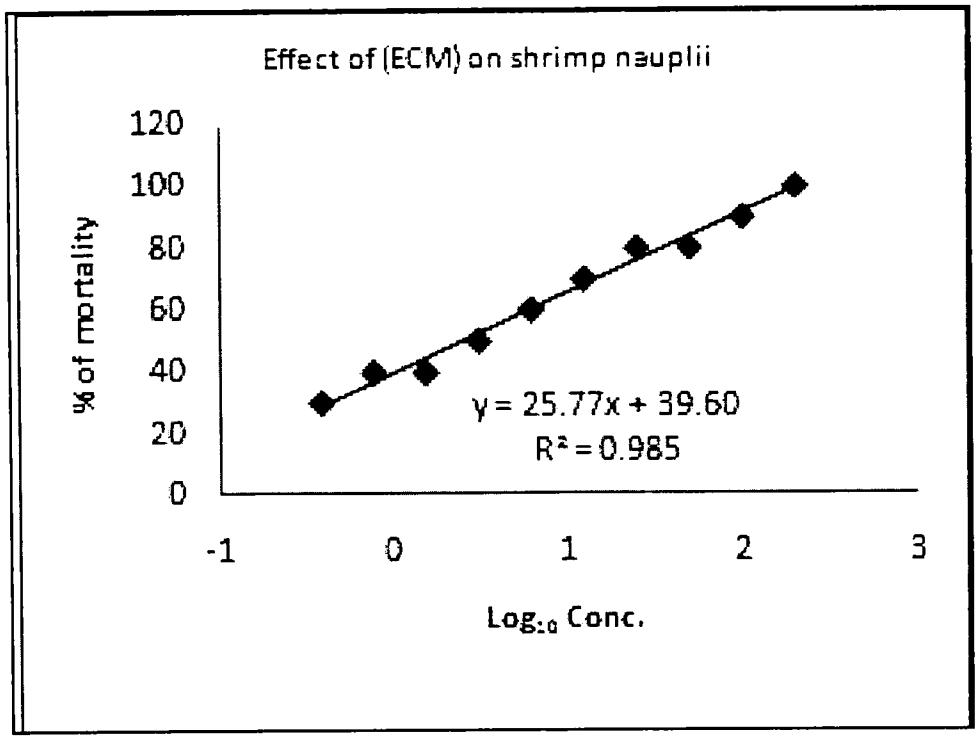

Fig: 3 Plot of mortality % and predicted regression line of ECM.

Table: 2.6 Effect of ethanolic extract of peel of Satkara (ECM) on shrimp nauplii.

Conc. (µg/ml)	Log_{10} conc.	% of mortality	LC_{50} (µg/ml)
0	-	00	2.53
0.390625	- 0.408	30	
0.78125	- 0.1072	40	
1.5625	0.1938	40	
3.125	0.4949	50	
6.25	0.7959	60	
12.5	1.0969	70	
25	1.3979	80	
50	1.699	80	
100	2	90	
200	2.301	100	

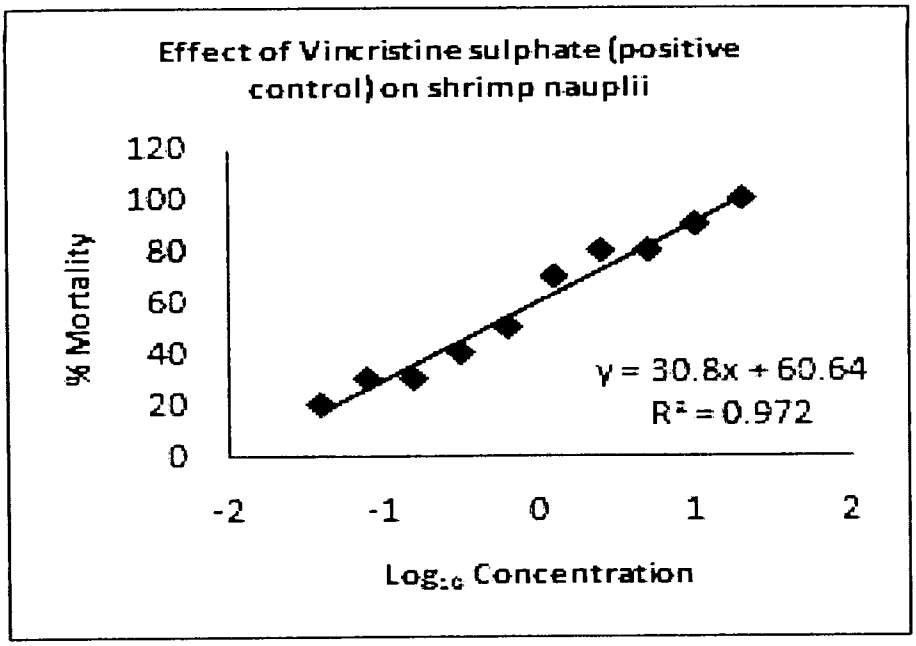

Fig: 4 Plot of mortality % and predicted regression line of VS.

Table: 2.7 Effect of Vincristine sulphate VS (positive control) on shrimp nauplii.

Conc. (µg/ml)	Log_{10} Conc.	% Mortality	LC_{50} (µg/ml)
00		20	0.451
0.0390	-1.4089	20	
0.078125	-1.1072	30	
0.15625	- 0.8061	30	
0.3125	- 0.5051	40	
0.625	- 0.2014	50	
1.25	0.09691	70	
2.5	0.39794	80	
5	0.6989	80	
10	1.00	90	
20	1.3010	100	

Table: 2.8 LC_{50} values of the test samples of Satkara.

Test samples	Regression line	R^2	LC_{50} (µg/ml)
ECM	y = 25.771x+39.609	98.51	2.53
VS	y = 30.8x + 60.645	97.29	0.451

ECM= Ethanolic extract of peel of Satkara
VS = Vincristine sulphate

F) Composition of the volatile oils of Satkara

The compositions of the volatile oil of the peel of Satkara are presented in Table2.9.

Table : 2.9 Compositions of the volatile oils of Satkara.

SL No	Compound	RRI[a]	%
1	Myrcene	1174	0.67 ± 0.12[b]
2	Limonene	1203	73.53 ± 2.54
3	1,8-Cineole	1213	tr[c]
4	γ-Terpinene	1255	0.37 ± 0.06
5	Octanal	1296	0.40 ± 0.10
6	Tridecane	1300	0.47 ± 0.12
7	Nonanal	1400	1.63 ± 0.06
8	trans-Linalool oxide (fuanoid)	1450	0.67 ± 0.15
9	cis-Linalool oxide (furanoid)	1478	0.43 ± 0.06
10	α-Copaene	1497	1.60 ± 0.10
11	Linalool	1553	1.17 ± 0.06
12	Octanol	1562	0.57±0.06
13	Terpinen-4-01	1611	0.30 ± 0.17
14	β-Caryophyllene	1612	1.97 ± 0.32
15	α-Humulene	1687	tr
16	Neral	1694	0.53 ± 0.06
17	α-Terpineol	1706	3.00 ± 0.27
18	Geranial	1740	tr
19	δ-Cadinene	1773	3.43 ± 0.15
20	Nerol	1808	tr
21	Geranial	1857	tr
22	*(E)*-Nerolidol	2050	1.43 ± 0.12
23	Elemol	2096	0.57 ± 0.21
24	γ-Eudesmol	2185	2.13 ± 0.21
25	Nootkatone	2554	2.13 ± 0.25
	Total		97.0 ± 2.62

a =RRI Relative Retention Indices calculated against *n*-alkanes on the HP Innowax column.b= mean % calculated from Flame Ionization Detector (FID) data ± SD (*n*= 3) c=(tr) trace (< 0.1 %)

The findings of the composition of the volatile oil of the peel are presented in Table 2.10. The distillation of 100 g fresh peels of the fruits of Satkara produced 120 mg of volatile oil (0.12%). The GC - MS analyses of the volatile oil led to the identification of 25 compounds accounting for over 97.0% of the total oil (Table 2.10). The majority of compounds present in the oil were monoterpenes. There were 15 monoterpenes, six of which possessed cyclic structure, e.g., limonene, 1,8-cineole, α-copaene, γ-terpinene, terpinen-4-ol and α-terpineol, and nine others had straight chain skeleton. Six sesquiterpenes, e.g. β-caryophyllene, α-humulene, δ-cadinene, elemol, γ-eudesmol and nootkatone were among 25 identified compounds.There were four nonterpinoidal alkane derivatives, e.g.octanal, tridecane, nonanal and octanol. Among the identified components, limonene was the major compound contributing about 74% of the total oil.

G) Biological activiies of volatile oil

Free radical scavenging activity

In the TLC based qualitative free radical -scavenging assay using the DPPH spray the volatile oil did not show any free radical scavenging property.

Antimicrobial activity

The results of antimicrobial activity of the volatile oil of Satkara are presented in Table 2.10.

Table : 2.10 Antibacterial activity of the volatile oil of Satkara peel.

Bacterial strains	Antibacterial activity (MIC mg/ml)	Ciprofloxacin in mg/ml
Bacillus cereus (ATCC 11778)	1.25	2.5×10^{-7}
Bacillus subtilis (NCTC 10400)	1.25	2.5×10^{-7}
Escherichia coli (ATCC 8739)	2.5	2.5×10^{-7}
Ampicillin-resistant *Escherichia coli* (NCTC 10418)	No activity	2.5×10^{-6}
Staphylococcus aureus (NCTC 1803)	5.0	2.5×10^{-8}
Salmonella typhii (NCTC 10203)	No activity	2.5×10^{-7}

The minimum inhibitory concentrations (MIC) of the volatile oil of extract against six bacterial strains were determined by the recently developed Resazurin microtitre assay. The volatile oil displayed reasonable antibacterial activity against four of the strains including, *Bacillus cereus, B.subtulis, Escherichia coli* and *Staphylococcus aureus,*, but not against amphicilin-resistant *E. coli* and *Salmonella typhii* (Table 2.11). The most potent activity was observed against both *Bacillus* species with an MIC value of 1.25 mg/ml. The antibacterial activity of the volatile oil was found to be bacteriostatic.

F) Qualitative test of some phytochemical constituents

Chemical tests were carried on the aqueous extract using the standard procedures to identify the some phytoconstituents of Satkara. The present study carried out to determine the presence or absence of medically active compounds. The phytochemical constituents are presented in the Table 2.11. Tannins, saponins, flavonoids, steroids, terpinoids and cardiac glycoside present in the peel samples of Satkara but alkaloid is absent.

Table: 2.11 Presence or absence of some phytochemical constituents of Satkara.

SL No	Name of the phytochemicals	Present(+)/ Absent (-)
1.	Tannins	+
2.	Saponins	+
3.	Alkaloids	-
4.	Flavonoids	+
5.	Steroids	+
6.	Terpinoids	+
7.	Cardiac glycoside	+

Chapter - 7

Discussion

Discussion

The nutritional compounds of citrus fruits were analyzed due to the present expanded consumption and production of fruits and increased demand for health. The citrus fruits and juices have several beneficial effect on health and nutritive properties (Okwu and Emenike, 2006. Rapisararda *et al.,* 1999).

Proximates

As mentioned earlier that Satkara is an exceptional fruits where mostly used the peels at mature and immature stage in cooking and pickle preparation. Till today so far we search that there is no nutritional analysis of this fruit to us.

Ash percentage were recorded (Bridsal *et al.*,1961) in California Lemon 0.61%, Valencia orange 0.78% and Neval orange 0.65% which is closed to present findings (0.89%). Similarly they reported moisture of peel for California lemon 81.62%, Valencia orange 85.05%and Neval orange 75.09% which is closed to present findings (75.53%) in *C. macroptera.*

Protein is an important chemical constituent of plants. Protein, fat, carbohydrate and energy from sweet orange were recorded 1.3%, 1.0%, 15.7% and 67 Kcal respectively. Bridsal *et al.,* (1961) also reported protein, and fat from California lemon are 1.47% and 0.32%, Valencia orange 1.13% and 0.23%, and Neval orange are 1.56% and 0.20%. Generally citrus fruits contain low fat and due to that it is ideal for good health (Roger,1999. Akobundu,1999.). In the present study Satkara contain low fat both in peel and juice, 0.85 g and 0.43 g respectively.

Citrus crude fiber has good medicinal value, softer stools and enhances frequent waste elimination, including bile acids, sterols and fats (Roger, 1999).The fiber when taken swells with water, this however increases volume in many times. This consequently gives consistency to the feces and facilitates its transit through the colon until it is expelled through the rectum. When diet contains low fiber then several problems including colon cancer may be developed ((Roger,1999). For detoxification of the body, humans need fiber (diatery fiber) in their diet, one such fiber rich fruit among citrus fruits

is lemon and is highly efficient when consumed raw. In a study conducted to know the benefits of lemon on disorders such as cholera, the antibacterial property of lemon was proven for *Vibrio* species of bacteria, which are the main causative organism for cholera (Tomotake *et al.*, 2006).In *C. macroptera,* the crude fiber was recorded only 2.26% for peel but average crude fiber of citrus (peel) species was reported 5.84 – 7.10%. The average energy value of fresh citrus is also low which can be very important for consumers concerned about putting on excess body weight. In Satkara both peel and juice contain low 90Kcal/100g and 83.23 g respectively.

The pH, TSS and Acids

Karadeniz, (2004) reported pH, Total soluble solids (TSS) and organic acids of different citrus species. These were pH for the juices, *Citrus reticulata* (Tangerine) 3.46, *Citrus sinensis* (Sweet orange) 3.34, *Citrus paradisi* (Grape fruit) 3.00 *Citrus.limon* (Lemon) 2.43 and *Citrus aurantium* (sour orange) 2.60. In Satkara (*C.macroptera*) peel found less acidic (4.43) but juice was very acidic (2.45) which are very close to lemon and sour orange. Karadeniz, (2004) also reported the TSS of *Citrus reticulata* (11.20%), *Citrus sinensis* (12.10%), *Citrus paradisi* (10.30%), *Citrus limon* (8.00%) and *Citrus aurantium* (10.00%) only. In Satkara, the TSS of peel was highest (24.47%) but juice contained 12.43% which was close to sweet orange and grape fruit, close to lemon and sour orange also.

Other than vitamin-C (ascorbic acid) citrus fruits also contain citric acid, malic acid and other acids. Citric acid is a weak organic acid and it is a natural preservative and is also used to add an acidic or sour taste to foods and soft drinks. Next to citric acid the malic acid present both in peel and juice. Citric acids found in the juices of fruits may be used as natural cosmetics and also for external application as a hair rinse, skin lotion and as a mouth wash (Randhawa & Srivastava, 1986). Karadeniz, (2004) reported citric acid and malic acid of the juices of Tangerine were 9.22 mg and 5.29 mg, Sweet orange 13.28 mg and 7.29 mg, Grape fruit 19.61 and 4.03, Lemon 55.11and 6.00, and sour orange 48.79 and 2.21. In Satkara citric acid from peel 4.53 mg and juice 55.25 mg were recorded. Compare with organic acids, Satkara is similar to Lemon and sour orange.

The Vit-C (ascorbic acid) in the fruit juices of different species of citrus has been recorded to variable and 25 mg to 85 mg/100 g (Ghosh, 1990). In Satkara, 53.07 mg of ascorbic acid was recorded for juice which is very handsome amount. As an antioxidant, Vit-C can prevent the cell damage done by "free radical" molecules as they oxidize protein, fatty acids and deoxyribonucleic acid (DNA) in the body. Free radical damage has been implicated in the progression of several diverse and important disease states including cancer, cardiovascular disease and cataract formation. Being a good source of antioxidants, if regularly consumed, citrus can be an important part of a diet aimed at reducing the risk of such chronic disease (Harats *et al.,* 1998).

Mineral nutrients

Beside acids the presence of micronutrients in this fruits indicates the good food and medicinal values. Okwu and Emanke, (2007) investigated the micronutrients of some citrus species which average values were Phosphate 0.24% - 0.4%, Sodium 0.28 - 0.36%, Potassium 0.28 -1.0%, Calcium 2.0 - 3.2% and Magnesium 0.49 - 0.61%. In our study Sodium and potassium were more higher (Na = 3.58% for peel and 2.57% for juice, K = 2.14% for peel and 1.14% for juice) but Ca is very less (Ca = 0.19% for peel and 0.21% for juice) than these findings.

A determination of minerals was carried out and the experimental results showed that some essential elements to humans such as calcium, magnesium, zinc, manganese, copper and sodium in grapefruit were abundant (Mandadi *et al.,* 2007). Along with Vit-C and citric acids citrus like other whole foods contain notable essential nutrients, like potassium, folate, calcium, thiamin, niacin, vitamin B_6, phosphorus, magnesium, copper and a variety of phytochemicals. Being a plant food it contains no cholesterol and low fat. Citrus fruits are important among populations who need to overcome and prevent micronutrient deficiencies as well as those concerned with problems of over-nutrition, obesity and diet-related chronic diseases (Economos, 1998).

In Satkara, the values of Cu, Zn, Fe, Mn were 0.33%, 0.13%, 0.07%, and 0.06%, for peel and 0.31%, 0.17%, 0.26%, and 0.05% for juice respectively.The results are almost

same for both peel and juice. In grape fruit, Soulis *et al.,* (2006) were Cu 0.17- 0.42, Zn 0.32 - 0.81, Fe 0.11 - 0.93 and Mn 0.04 - 0.82% for juice which were higher than Satkara juice.

Antimicrobial activity of peel extracts

Plants are the natural reservoir of many antimicrobial agents. In recent times, traditional medicine as an alternative form of health care and to overcome microbial resistance has led the researchers to investigate the antimicrobial activity of medicinal plants (Austin *et al.,* 1999).The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility to various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth.

Antimicrobial activity was evaluated by disc diffusion method (Bayer *et al.*, 1966). It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials (Ronald, 1982). In this present study, the zones of inhibition produced by the crude ethanolic extracts of satkara were ranged of 8 - 9 mm at a concentration of 300 µg/disc. None of the Gram positive bacteria except *Staphylococcus aureus* showed any zone of inhibition. Whereas in case of Gram negative bacteria only *E.coli,Vibri mimicus* and *Shigella boydii showed* 8 mm zone of inhibition. All the fungi showed 8 - 9 mm zone of inhibition. Gulay *et al.,* (2009) reported zone of inhibition for *E.coli* in grapefruit 10, orange 13, and mandarin 12, while for *Staphylococcus aureus* in grapefruit 11, orange 12 and mandarin 14. The results were almost similar with present study.

Singh *et al.,* (2009) evaluated the antibacterial activity of oil of *C. sinensis* by disc diffusion method against *Bacillus subtilis* and *Escherichia coli* using erythromycin as a standard and DMSO as a control sample. Zone of inhibition of *E.coli* and *B. subtilis* was 13 mm and 17 mm respectively when compared with the results of standard i.e. 31 mm against *E. coli.* and 32 mm against *B. subtilis.* Therefore, in the future several citrus including Satkara based phytoconstituents may be used in treating certain life threaten diseases. Taiwo *et al.*, (2007) investigated the antibacterial effects of *Citrus*

aurantifolia L(lime) commonly used as traditional medicines in Nigeria. The antibacterial activities of aqueous extracts of this plant was evaluated using disk diffusion susceptibility testing on 53 fresh human bacterial pathogens and they found promising broad spectrum antibacterial effects on human pathogens.

Brian shrimp lethality assay

Brine shrimp bioassay is a rapid general bioassay for the bioactive compound of the natural and synthetic origin (Meyer *et al.*, 1982, Persone,1980). Bioactive compounds are almost toxic in higher doses. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and pharmacological activities of natural products. By this method, natural product extract, fraction as well as the pure compounds can be tested their activity.

According to McLaughlin, (1991) brine shrimp lethality bioassay is indicative of cytotoxicity and a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor activity of the compounds.

From the results of the brine shrimp lethality bioassay, it can be well predicted that the crude extract of Satkara possess good cytotoxicity principles. Compared with positive control (VS) the cytotoxicity exhibited by the extracts indicated that further bioactivity guided investigation can be done to find out potent active compounds from this plant material for evaluation of anticancer, antitumour and other pharmaceutical activities.

Components volatile oil

Ahamad *et al.,* (2006) mentioned that the peel of citrus fruits is a rich source of flavanones and polymethoxylated flavones, which are very rare in other plants. These compounds not only play a important physiological and ecological role, but are also of commercial interest because of their multitude of application in the food and pharmaceutical industries. Naringin and hesperidin have many biological activities such as antioxidant antimutagenic effect, analgesic ,antiflamatory etc.

Certain chemical compounds known as limonoids have been shown to prevent the occurrence of cancers of the breast, skin, lung, mouth, stomach and colon in animal studies and laboratory tests with human cells. limonin are considered to be highly potent in preventing the spread of cancer owing to their increased availability in our body for a prolonged duration after being absorbed (Manners, 2007)

Steinmetz and Potter, (1991) reported that naturally occurring compounds found in plants have a wide range of physiological effects and may help to protect against various chronic diseases, including cancer and heart disease. The wide variety and number of known phytochemicals continue to grow, as does understanding of their role and importance in the diet. Several classes of phytochemicals, including monoterpenes, limonoids (triterpenes), flavanoids, carotenoids and hydroxycinnamic acid, have been isolated from citrus. Citrus peel contains a range of essential oils. These essential oils are found to inhibit the growth of or kill bacteria which are pathogenic in nature. Citrus peels also possess diabetes lowering and antiperoxidative effect due to the high content of total polyphenols (Nannapaneni *et al.,* 2008).

Belletti *et al.,* (2004) in their experiment found that the most effective essences were characterized by the highest concentration of some terpenes, such as citral, β-pinene, and *p*-cymene. Moreover, all of the essences were more bioactive when added directly to the liquid medium. Bhuiyan *et al.,* (2009) analyzed the chemical constituents of leaf and peel essential oil of *Citrus medica* L. by Gas Chromatography (GC) and Gas Chromatography and Mass Spectroscopy (GC - MS). Ninteen components accounting for 99.9% of the total oil are found in leaf oil. The major components are erucyclamide (28.43%). Limonene (18.36%) and citral (12.95%). The peel oil contains 43 components accounts for 99.8% of the total oil and the major components are isolimonene (39.37%), citral (23.12%) and limonene (21.78%).

As a part of this research work the compononents of volatile oils of Satkara (*Citrus macroptera* Mont.Var. *annamensis*) was analyzed and obtained 25 compounds (Miah *et al.,* 2010). Among them Limonene was found 73.53%. Hamadan *et al.,* (2010) analyzed the essential oils obtained from the fruit peels of *Citrus jambhiri* L (Rough lemon) and *C. pyriformis* Hassk (Ponderosa lemon) A total of 94 compounds were unambiguously identified from the oils and the (hexane/ether) extracts of the peel and

juices representing 98.55% and 97.98% of the total oil composition. The main component of both oils was D – limonene (92.48% and 75.56% respectively).

Free radical scavenging activity of volatile oil

The DPPH antioxidant assay is based on the principle that 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, can be decolorized in the presence of free radical scavangers. The odd electron in the DPPH radical is responsible for the absorbance at 517 nm, and also for visible deep color (Kumarasamy *et al.,* 2007). When DPPH accepts an electron donated by antioxidant compound, the DPPH is decolorized. In the present study, volatile oil from peel did not show any free radical scavenging property, which was evident from any white/yellowish-white spots against the pink background of the TLC plate sprayed with DPPH.

Antimicrobial activity of volatile oil

Singh *et al.,* (2009) observed that due to increasing concerns about the development of antimicrobial resistance amongst pathogenic bacteria, alternative strategies have been sought that do not use antibiotics to reduce pathogenic bacteria from foods and patients. A natural compound that has potent antimicrobial properties is citrus peel, which contains a variety of essential oils that inhibit the growth or kill of pathogenic bacteria.

The volatile oil of Satkara peel displayed reasonable antibacterial activity against four of the strains including, *Bacillus cereus, B. subtulis, Escherichia coli* and *Staphylococcus aureus,*, but not against amphicilin-resistant *E. coli* and *Salmonella typhii.* The most potent activity was observed against both *Bacillus* species with an MIC value of 1.25 mg/ml (Miah *et al.,* 2010). The antibacterial activity of the volatile oil was found to be bacteriostatic. The antibacterial activity profile displayed by the volatile oil of Satkara was similar to that observed with *Citrus sinensis* volatile oil (Njoroge *et al.*, 2009).

Phytonutrients

The present study carried out to determine the presence or absence of medically active compounds. The phytochemical constituents are tannins, saponins, flavonoids, steroids, terpinoids and cardiac glycoside present in the peel samples of Satkara but alkaloid is absent.

Oleuremi *et al.,* (2007) experimented the peels of four varieties of citrus fruits namely *Citrus sinensis* (Washington), *Citrus sinensis* (Ibadan), *Citrus limonum* and *Citrus qurantifolia* were sun-dried and milled to obtain citrus fruit peel meal (CFPM). The CFPM of each variety was subjected to phytochemical screening to detect the presence of phytonutrients. Phytochemical screening showed that each of the peels contained tannin, saponin, phytate, oxalate, flavonoids and limonene. Whereas, the variation of each of these phytonutrients between the peel meals was significantly different ($p<0.01$) except for tannin ($p>0.05$), they were at low concentrations and below the levels reported in literature to be toxic to livestock species.

Constituents of Satkara indicated the medicinal importance of this fruit. Tannins, saponins, flavonoids, terpinoids, steroids and cardiac glycosides were known to show medicinal activity as well as exhibiting physiological activity (Sofowara,1993). Tannins have shown potential antiviral (Lwu *et al.,* 2004) and antibacterial (Akiyama *et al.,* 2001) activity. Tannins can also be effective in protecting the kidneys and have been used for immediate relief of sore throats, diarrhea, dysentry, skin ulcers etc. There is evidence of the presence of saponins in traditional medicine preparations (Xu *et al.,* 1996). Plant terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for antibacterial and other pharmaceutical functions. Steroids are medicinally so important for increasing strength of human body. According to Manners, (2007) flavonoids have been believed to have anticancer properties. Flavonoids are sometimes called "nature's biological response modifiers" because of their anti-inflammatory, antiallergenic, antiviral, antioxidant and their anticancer properties.

Chapter - 8

Conclusion

Conclusion

Because of certain phytochemical characteristics relating to health benefit, there has been rapid increase of the consumption of *Citrus macroptera* var. *annamensis* (Satkara) fruit in culinary and pickle preparation.

Results on the proximate values clearly indicated the food value of Satkara. It is not only rich in calories but also contain adequate quantity of protein and fiber. It has also contained low amount of fat which is highly beneficial to health to reduce obesity and overweight.The juice of Satkara has good source of Vit-C and high amount of citric acid but low amount of malic acid. Vit-C is a water soluble and it is needed daily (60 mg) to maintain the skin and teeth, to resist stress, infection and scurvey. Vit-C is easily deactivated by cooking or standing in the presence of air. Citric acid is a natural preservative and used to add an acidic or sour taste to foods and soft drinks. Due to the presence citric acids in peel Satkara used in curry for sour taste and supposed to soften the meat and fish.

Both peel and juice contained the mineral nutrients like P, Ca, Mg, K , Na, Cu, Mn, Zn and Fe. The presence of high amount of Na and K in juice is very helpful to recover the electrolyte balance of the body. It is also important for the person who needs to overcome and prevent micronutrient deficiencies as well as those concerned with problems of over-nutrition, obesity and diet-related chronic diseases.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials.The ethanolic extract of the peels (ECM) were subjected to brine shrimp lethality bioassay. The lethal concentration LC_{50} value of ECM was found to be 2.53 μg/ml, which is compared to reference standard VS with LC_{50} of 0.45 μg/ml following regression analysis. Brine shrimp lethality bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid in process, inexpensive and requires no special equipment or aseptic technique. It utilizes a large

number of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

The antibacterial activity of the ethanolic peel extracts of Satkara was evaluated using disc diffusion method. Both Gram positive and Gram negative bacteria and fungi were used in this experiment. None of the Gram positive bacteria except *Staphylococcus aureus* showed any zone of inhibition. Whereas in case of Gram negative bacteria only *E. coli, Vibrio mimicus* and *Shigella boydii showed* 8 mm zone of inhibition. All the fungi showed 8 - 9 mm zone of inhibition. The overall results indicated the less antimicrobial activity of the peel extracts.

Volatile oil collected through steam distillation were analyzed both by gas chromatography (GC) and gas chromatography-mass spectrometry (GC - MS) of the sample resulted in the identification of 25 terpenoids, predominantly monoterpene hydrocarbons, accounting for 97% of the total oil, and limonene (73.5%) as the major component. Next to limonene, δ-Cadinene (3.43%), α-Terpineol (3.00%), γ-Eudesmol (2.13%) and Nootkatone (2.13%) were evaluated. These terpinoids are very important our health related problems supported by several researchers. The essential oil has also a high quality flavor observed during study.

The volatile oil of Satkara peel displayed reasonable antibacterial activity against four of the strains including, *Bacillus cereus, B.subtulis, Escherichia coli* and *Staphylococcus aureus,*, but not against amphicilin-resistant *E. coli* and *Salmonella typhii.* The oil did not exhibit any *in vitro* free-radical - scavenging (DPPH) properly.The most potent activity was observed against both *Bacillus* species with an MIC value of 1.25 mg/ml. The antibacterial activity of the volatile oil was found to be bacteriostatic.

Chemical tests were carried out on the aqueous extract of fresh peels of *Citrus macroptera* using the standard procedures to identify the some phytoconstituents. In the present study determined the presence or absence of medically active compounds. The phytochemical constituents like tannins, saponins, flavonoids, steroids, terpinoids and cardiac glycoside were present in the samples but alkaloid was absent.

PART - III

IN VITRO PROPAGATION

Chapter - 9

Materials and Methods

IN VITRO PROPAGATION

Materials and methods

A) Surface sterilization of explants from field grown plants of Satkara.

Materials

Materials were collected from Citrus Research Station of Bangladesh Agricultural Research Center (BARI), Jaintapur, Sylhet during 2009 and 2010. Some grafted plants were grown in earth pots at Botanical garden, Department of Botany, University of Dhaka.

Surface cleaning and sterilizing chemicals

In this investigation 1% Savlon, 70% ethyl alcohol, Tween-twenty (T-20), 5% Sodium Hypo chlorite Solution ($Na(OCl)_2$, and 0.1% $Hgcl_2$.

Methods

The following five procedures were followed for surface sterilization of explants materials.

Procedure – 1(P_1)

In the field

Freshly growing stems of 7 to 8 weeks old were selected from field grown plants for the explants of experiment. About 20 - 25 cm long stem cut with leaves and cut end dip into the 5% $Na(OCl)_2$ solution for few seconds to stop the entry of new microbes. Then stems were washed with normal water for few minutes to eliminate the dirt particles. Stems were then washed with mineral water and detergent powder (Jet powder) followed by distilled water. Then the stems were kept in poly bag (Plate 3.1 a) and a small piece of ice rapped with cotton and

placed at the bottom of the bag to maintain the moisture for 16 –18 hrs. The bag was air pumped and tightly bound by threat.

In the laboratory

Materials were brought in the laboratory of Department of Botany, University of Dhaka to complete the rest part of sterilization. Stems were defoliated and after washed with distilled water cut into convenient sizes and taken into a beaker and again washed with detergent powder and liquid (Savlon).Then dipped into 70% alcohol for 3 minutes and washed with distilled water and next dipped into 0.1% $HgCl_2$ solution and added 2 - 3 drops of T -20 and kept for 10 minutes under laminar air flow cabinet. Explants were then washed with sterilized distilled water for 6 times. Thus the explants were sterilized and ready for culture.

Procedure –2 (P_2)

In the field

Collection of stem branches and transported from Sylhet to Dhaka was same as P_1, but in laboratory the sterilizing chemical is different.

In the laboratory

All the steps were same as P_1, except 5% Na $(OCl)_2$ was used instead of 0.1% $HgCl_2$ for 10 minutes.

Procedure – 3 (P_3)

In the field

Freshly growing stems of 7 to 8 weeks old were selected from the mature field and pot plants for experiment. About 10 - 12 cm long stem branches cut with leaves and cut end dip into the 5% $Na(OCl)_2$ solution for few seconds to stop the entry of new microbes. Then stems were washed with normal water for few minutes to eliminate the dirt particles. Stems were then washed with mineral water and detergent powder (Jet powder) followed by distilled water. Then the stems were kept in poly bag (Plate 3.1 c) and a water soaked cotton piece

placed at the bottom of the bag to maintain the moisture for 16 –18 hrs. The bag was air pumped and bound tightly.

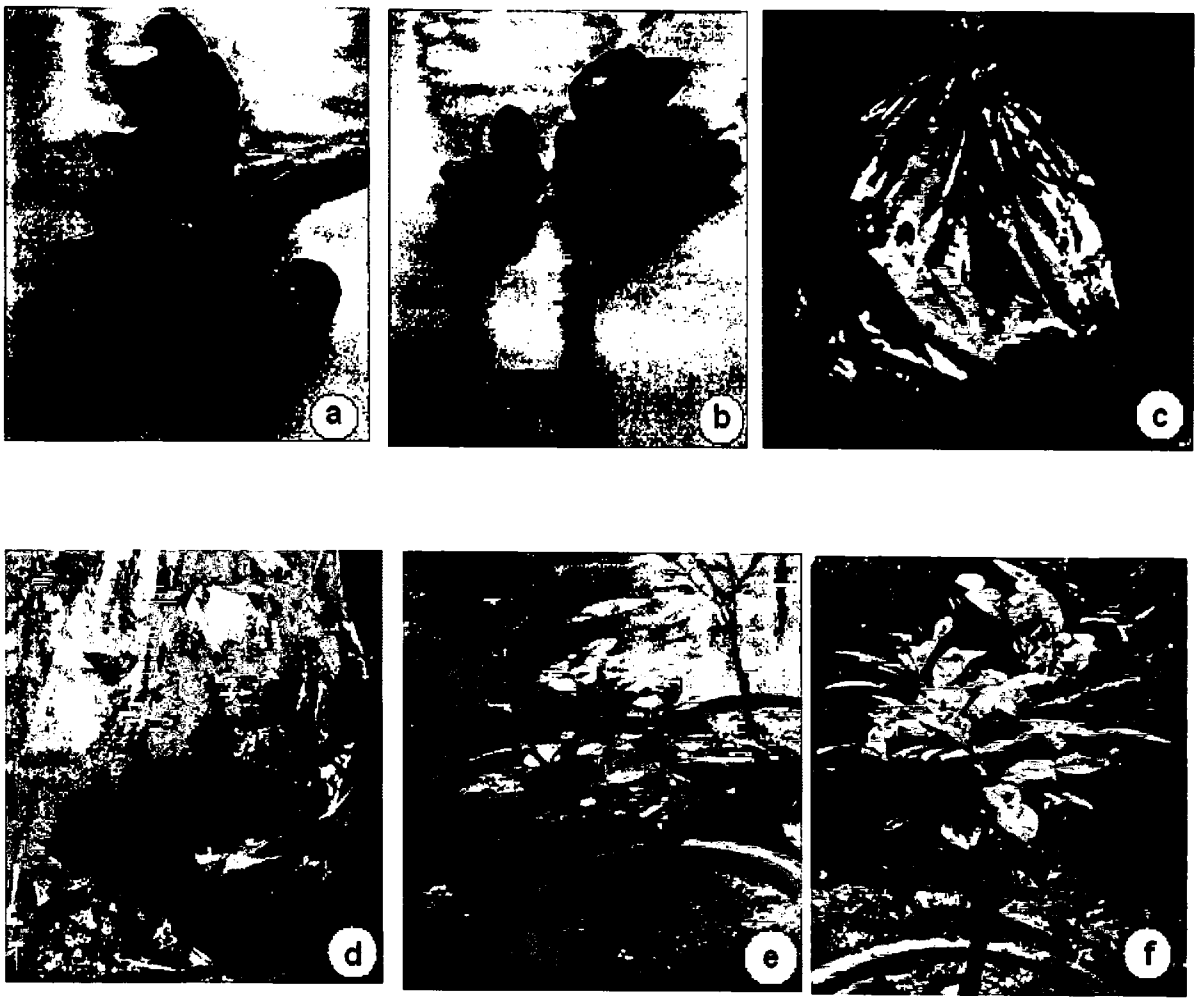

Plate: 3.1 Explant sources of Satkara germplasm (a) & (b) stem branch with leaves (c) & (d) stem branches within poly bag (e) pot plant grown in botanical garden of Department of Botany, University of Dhaka under nylon net (f) pot plant grown in open field.

In the laboratory

Explants were brought in the laboratory of Department of Botany, University of Dhaka in next day and completed the rest part of sterilization. Stems were defoliated and after washed with distilled water cut into convenient sizes and taken in a beaker. Then washed with 2 - 3 drops of T - 20 solution, detergent powder and liquid (Savlon) and washed with distilled water and dipped into 70% alcohol for 2 minutes and then dipped into 0.1% $HgCl_2$ solution for 15 minutes under laminar air flow cabinet. Explants were then washed with sterilized distilled water for 6 times. Thus the explants were ready for culture.

Procedure – 4 (P_4)

In the field

Collection of stem branches and transportation from Sylhet to Dhaka was same as P_3.

In the laboratory

All were same as P_3, except 5% Na $(OCl)_2$ was used instead of 0.1% $HgCl_2$ for 15 minutes

Procedure –5 (P_5)

In the field

Collection of stem branches and transportation from Sylhet to Dhaka was same as P_3.

In the laboratory

Explants were brought in the laboratory of Department of Botany, University of Dhaka to complete the rest part of sterilization. Stems were defoliated and after washed with distilled water cut into convenient sizes and taken into a beaker. Then rinsed with 2 - 3 drops of T - 20 solution, detergent powder and liquid (Savlon) and washed with distilled water and dipped into 5% Na $(OCl)_2$ for 10 minutes. After washed with distilled water explants were again dipped into 0.1% $HgCl_2$ for 10 minutes and washed with sterilized distilled water for 6 times. Finally dipped into 70% alcohol for 30 seconds and explants were washed with

sterilized distilled water and kept on sterilized filter paper to soak the water. Thus the explants were sterilized and ready for culture.

Transfer of explants

The stems were cut into 10 -12 mm length with a single axillary bud by sharp scalpel under laminar airflow cabinet. Then single explants were cultured on MS basal media (MS_O) and MS supplemented with BAP1mg/l (MS_1) media. Conical flask (150 cc) and test tubes (50 cc) were used as culture vessels.

Media preparation

Preparation of stock solutions

For convenience, separate stock solutions for macronutrients, micronutrients, Fe-EDTA, vitamins and amino acids, plant growth regulators etc. were prepared for ready use.

Culture medium formulated by Murashige and Skoog – MS (1962) was used in this work.

(i) Stock solution of macronutrient (500ml)

Stock solution of macronutrient was made in the following way:

1) Its strength was twenty times of (× 20) final concentration in 500 ml distilled water solution (Appendix - 1).
2) For this purpose, 10 times the weight of different salts present for ½ liter of the medium were weighted (Appendix -1)
3) The salts were dissolved one after another in 400 ml of distilled water and then volume was made up to 500 ml.
4) The solution was then filtered through filter paper (What men No. 1) to remove all the solid contaminants like dirt, foreign bodies etc.
5) The stock solution of macronutrients thus prepared and was stored in refrigerator at 4°C for a 6 - 8 weeks period until the solution was totally used.

(ii) Stock solution of micronutrients (250ml)

Its strength was 200 times that of (× 200) final concentration of the medium in 1 liter of the distilled water as described for the stock solution to micronutrients (Appendix 1). In case of 250ml stock (× 200) 50 times the weight of different salts were weighted (Appendix - 1)

Two separate stock solutions of micronutrients were prepared as follows:

(iii) Stock solution of micronutrients (except $FeSo_4$ and Na_2-EDTA)

1) For this purpose, 50 times the weight of different micronutrient salts present for 250ml of the solution were weighted (Appendix 1)

2) The salts were dissolved separately and poured into a beaker containing 150 ml of distilled water and then volume was made up to 250 ml.

3) The solution was then filtered through filter paper (What men No. 1) to remove all the solid contaminants like dirt, foreign bodies etc.

4) The stock solution of macronutrients thus prepared and was stored in refrigerator at 4°C for several weeks until the solution was totally used.

(iv) Stock solution of $FeSo_4$ and Na_2-EDTA

At first both Na_2-EDTA and $FeSo_4.7H_2o$ was weighted 50 times of the original weight (Appendix-1). Then first dissolved Na_2-EDTA in 100 ml of boiling distilled water, and then $FeSo_4.7H_2o$ was added gradually. Then solution kept of magnetic water bath with constant heat and stirring for at least 1 hr until the color of solution changed into golden yellow. Finally the volume was made up to 250 ml and solution was filtered and then glass bottle raped by aluminum foil and kept in refrigerator at 4 - 5° C for several weeks.

(v) Stock solution of vitamins and amino acids

The following vitamins and amino acids were used in MS medium (Appendix 1).

Pyridoxine HCl (Vitamin B_6)

Thiamine HCl (Vitamin B_1)

Glycine

Nicotinic acid (Vitamin B_3)

This was made 200 times of the final strength of vitamins and amino acids in 250 ml of distilled water. Each of the recommended ingredients weighed 50 times of the original (Appendix -1) then ingredients were dissolved separately and then was mixed in a beaker. The final volume was made up to 250 ml and poured into glass bottle, labeled and stored in refrigerator at 4°C for 15 - 20 days.

Myo-inositol was directly added to the medium during preparation in desired concentration i.e. 100 mg/l (0.1 g). Sucrose or cane sugar which ever required (30 g/l) was added directly during the preparation of medium. Agar powder (8.5 g/l) also added directly before autoclaving.

(vi) Stock solution of growth regulators

In addition to the nutrient, it is generally necessary to add one or more growth regulators such as auxins, cytokminins to the media to support good growth of tissues and organs (Bhojwani and Razdan, 1983). Separate stock solution was prepared for each growth regulator by dissolving it in a very minute quantity of the appropriate solvent and making up the final volume with distilled water.

The following hormonal supplements were used in the present investigation:

1) Auxins

Indole-3-butyric acid (IBA)

α-naphthalene acetic acid (NAA)

2) Cytokinins

6-benzyl aminopurine (BAP)

Kinetine (Kn)

The growth regulators and additives were dissolved in appropriate solvent as shown against each of them (following the Sigma Plant Cell Culture Catalogue 1992).

Hormones (solutes)	Solvents
IBA	70% Ethyl alcohol
NAA	0.1 N NaOH
BAP	0.1 N NaOH
Kn	0.1 N HCl

To prepare the stock solution of any of the hormones, 20 mg (0.02 g) of solid hormone placed on a clean watch glass and then dissolved in 1 - 3 ml of particular solvents. The mixture was then washed off by distilled water and collected in a 100 ml measuring cylinder and was made up to 100 ml by further addition of distilled water. The solution was then poured into a glass container, labeled and stored at 4°C for up to 4 - 5 weeks.

Preparation of MS culture medium

To prepare 1 liter of MS medium, the following steps are to be followed:

i) 30 g of cane (market) sugar was dissolved in 500 ml of distilled water.

ii) In another beaker 200 ml of distilled water was taken and then stock solutions (Macronutrient 50 ml, micronutrient 5ml, Fe-EDTA 5 ml vitamins 5 ml) were added sequentially and mixed well.

iii) 100 mg (0.10 g) of Myo-inositol was directly added to this solution and dissolved completely.

iv) Different desired concentration of hormonal supplements were added to this solution either individually or in combination and mixed thoroughly. Since each 100 ml of hormonal stock solution contained 20 mg hormonal salts, so addition of 5 ml of any hormonal stock solution to make 1 liter of medium result 1 mg/L concentration. Similarly, 0.5, 1.0, 1.5 ml may contribute o.1, 0.2 and 0.3 mg/L concentration respectively.

v) The whole mixture was then taken into a measuring cylinder and made up to 1 liter volume with further addition of distilled water and transferred into a one-liter beaker and mixed well. In case of half MS media preparation the ingredients were added according to their desired amounts where needed (Appendix - 2).

vi) The pH of the medium was adjusted to 5.7 ± by the help of pH meter with the aid of 0.1 N HCl and 0.1 N NaOH, whichever it was necessary.

vii) For solidifying the medium 8.5 g of agar (BDH) was added to this solution and mixed well by magnetic stirrer on magnetic bath and then the whole mixture was gently heated in a microwave oven till the dissolution of agar.

viii) Fixed volume of hot medium was dispensed into culture vessels, viz. test tubes, conical flasks. The test tubes or conical flasks were plugged with aluminum foil. The vessels were marked with different codes by the help a glass marker to indicate specific hormonal supplements.

ix) The culture vessels containing medium were autoclaved at 15 –lbs/sq inch pressure and at the temperature of 121°C for 20 minutes to ensure sterilization.

Preparation to ensure aseptic culture

Aseptic condition is always prerequisite for *in vitro* culture. Laminar airflow cabinet is the most suitable, convenient and reliable instrument for aseptic work. So, all inoculations and aseptic manipulations were carried out in Laminar airflow cabinet.

i) The cabinet was kept on for 30 minutes before use and cleaned with 70% ethyl alcohol to reduce the chances of contamination.

ii) The instruments like scalpel, forceps and requirements like Petri dishes, filter paper, empty beaker and conical flask, cotton, distilled water, which were covered with Aluminum foil and autoclaved by steam sterilization method. During working time the dissection instruments were again sterilized by an alcoholic dip (on 90% alcohol) and flamed over a spirit lamp for several time.

iii) A clean apron and mask were used during aseptic works.

iv) Hands were also sterile so far as possible by spraying 70% alcohol.

v) Surgical operations were taken care as usual to obtain possible contamination free condition.

Inoculation / Transfer

The laminar airflow cabinet was started 30 minutes before the inoculation work. All the sterilized articles (media, instruments, glass goods etc.) were put on the table of laminar airflow cabinet for inoculation.

B) Seed germination and *in vitro* micrografting

In vitro seed germination technique was undertaken to ensure contamination free seedlings which were used as rootstocks for microshoot grafting. Seedlings of pummelo were used as rootstocks for micrografting. The seeds were decoated before surface sterilization. Following the procedure -5 (P_5), the surface sterilization of seeds was done. Then the seeds were inoculated in MS basal medium . *In vitro* grown 5 - 6 weeks old seedlings containing vessels sprayed with 70% alcohol and they were transferred to a sterile petridish with the help of sterile forceps and were used in micrografting.

Buds (2 – 3 mm) from *in vitro* grown explants were separated by sharp scalpel and fixed in rootstocks by top incision method with the help of forceps, sharp blade and hand magnified lance. The joint of the graft was tightly bound by grafting tape or aluminum foil. Then the grafted plant was inoculated into MS media supplemented with BAP 1mg/l + NAA 0.5 mg/l. After inoculation the culture vessels were plugged by aluminum foil and labeled by glass marker pen. After 4 weeks when bud settled then rootstock was transferred into pot soil in growth room. Initially the pots were covered by polythene and gradually within 15 days the bag was removed.

C) Micrografting and *ex vitro* growth

In case of *ex vitro* micrografting the buds (3 – 4 mm) from *in vitro* grown explants were separated by sharp scalpel and fixed in *ex vitro* grown rootstocks by side grafting or top incision with the help of forceps and sharp blade. The joint of graft was also bound by grafting tape and plant grown in pot soil in growth room. Initially the pots were covered by polythene and gradually within 15 days the bag was removed.

D) Regeneration of shoots from nodal explants

Collection of explants

Stem branches of 6 - 7 weeks old pot grown plants of botanical garden of Department of Botany, University of Dhaka were collected during 2010.

Surface sterilization

At first stem branches were washed 30 minutes with running tap water. According to P_5, (sterilizing procedure) stems were defoliated and after washed with distilled water cut into convenient sizes and taken into a beaker. Then rinsed with mixture of 2 - 3 drops of T -20 solution, detergent powder and liquid (Savlon) and washed with distilled water. Next dipped into 5% $Na(OCl)_2$ for 10 minutes. and after washed with distilled water explants were again dipped into 0.1% $HgCl_2$ for 10 minutes and washed with sterilized distilled water for 6 times. Finally dipped into 70% alcohol for 30 seconds and explants were washed with sterilized distilled water and kept on sterilized filter paper to soak the water. Thus the explants were sterilized and ready for culture.

Inoculation

Explants were placed on petridish and cut into 10 -12mm size with a single node. Then carefully transferred into BAP supplemented MS media and vessels were plugged by aluminum foil.

Incubation

Explants were kept in a rack of growth room where light, temperature and humidity under controlled. The cultures were maintained at 25 - 27°C under the

cool white fluorescent lights having light intensity varied from 2000-3000 Lux for 16 hr photoperiod. The culture vessels were checked regularly for observation..

Root induction in regenerated shoots

The regenerated shoots were very carefully separated from the culture vessels and placed on a sterilized petridish and the basal ends of the shoots were cut. Then.shoots were cultured in hormone free MS medium for further elongation. The elongated shoots after 3 weeks were directly transferred to sterilized plastic pots filled with sterile soil and wood dust compost in the ratio of 1:1 for adventitious root formation.

Transplantation

After sufficient growth of shoot in MS medium they were transferred into pot soil for direct rooting.

In case of regenerated shoot the following procedures were followed for *ex vitro* rooting.

1. Generally when shoots were became 15 – 20 mm lengths with 3 - 4 leaves, these were then ready for transplantation.
2. The shoots were carefully removed from culture vessels. Agar attached to the basal part was gently washed out with distilled water. Then shoots were transferred to white plastic pots filled with soil and compost (1:1).
3. The pot soil sprayed with (0.5% w/v) Benolate fungicide.
4. After transfer the shoots were covered with transparent polyethylene bags to prevent desiccation. For aeration the bag was perforated at the top right corner.
5. The plastic pots were kept in growth room under high humid condition with light (2000 Lux for 16 hr photoperiod). The pots were regularly checked up and interior of the bag was sprayed with water at every 24 hrs.
6. After 3 - 4 days the bags were transferred to hardening room (net house).

7. The polyethylene begs were gradually more perforated to expose the plants to outer environment. This practice facilitates gradual acclimatization of the transplants to the outer stress environment.

8. By this time new leaves emerged and plantlets became established in the soil and they began to show a good performance.

Data collection

Data were collected under the following headings,

(a) Direct shoot regeneration

Number of explants inoculated, percent of explants responded, days to shoot initiation, percentage of explants developed multiple shoot, number of usable shoots per culture and shoot length (cm).

(f) Direct (*ex vitro*) root induction

Number of shoots inoculated, percentage of shoots in which root developed, days to root initiation and length of roots (mm).

Chapter - 10

Results

Results

A) Surface sterilization

The results and observations of the experiment are presented in Table 3.1. Five sterilization procedures were followed. Explants collected from Sylhet and pot grown plants in Botanical garden of Department of Botany were used in five procedures ($P_1 - P_5$). Explants were cultured in both conical flask and test tubes (Plate 3.2 a & b). The data were recorded after 5 weeks of culture. The rate of contamination and characteristics of contaminations are also presented in Table 3.1 & Plate 3.2.

Limited number of explants was used in MS_O media for five procedures. No sprouting of shoots was recorded in all explants cultured on MS_O medium. In case of P_1, 100% contamination was recorded for field explants and 80% for pot explants in MS_0 medium. 20% shoots regenerated in MS_1 medium, but contaminated later i.e. contamination rate was 100% for both field and pot explants.

In case of P_2, 100% contamination was recorded for both field and pot explants in MS_0 medium. The contamination rate was 100% for field explants in MS_1 and no sprouting of shoot was recorded. 40% of shoots regenerated in MS_1 medium but contaminated later (Plate 3.2 g & h).

In P_3, 100% contamination was recorded for both field and pot explants in MS_0 medium. The contamination rate was 100% for field explants in MS_1 and no sprouting of shoot was recorded. 20% of shoots regenerated in MS_1 medium but contaminated later.

In P_4, 100% contamination was recorded for field explants and 80% for pot explants in MS_0 medium. 20% shoots regenerated in MS_1 medium ,but contaminated later i.e. contamination rate was 100% for field and pot explants.

The best results were recorded in P_5, where no contamination for field explants but, 20% contamination for pot explants was recorded in MS_O medium. The 80% of pot explants and 30% of field explants were sprouted in MS_1 medium. Finally 80% of shoots were found contamination free for pot explants but shoots from field plants later contaminated.

Table: 3.1 Procedures of surface sterilization and rates of contamination of Satkara explants.

Procedures of sterilization	Media and number of explants used	% of explants responded for shoot induction	% of explants contaminated	Contamination % of regenerated shoots	% of non contaminated shoots
P_1	MS_O = St-5	00%	100%	----------	-------
	Pt-5	00%	80%	---------	-------
	MS_1= St-10	00%	100%	----------	--------
	Pt-5	20%	100%	100%	--------
P_2	MS_O = St-5	00%	100%	----------	-------
	Pt-5	00%	100%	---------	-------
	MS_1= St-10	00%	100%	----------	--------
	Pt-5	40%	100%	100%	-------
P_3	MS_O = St-5	00%	100%	----------	-------
	Pt-5	00%	100%	---------	-------
	MS_1= St-10	00%	100%	----------	--------
	Pt-5	20%	100%	100%	--------
P_4	MS_O = St-5	00%	100%	----------	-------
	Pt-5	00%	80%	-------	-------
	MS_1= St-10	20%	100%	100%	--------
	Pt-5	20%	100%	100%	--------
P_5	MS_O = St-5	00%	00%	----------	-------
	Pt-5	00%	20%	---------	-------
	MS_1= St-10	30%	100%	----------	--------
	Pt-10	80%	20%	00%	80%

MS_O = MS basal medium.; MS_1 = BAP 1mg/l supplemented MS medium:
St = Explants collected from Sylhet: Pt = Explants collected from pot plants grown in Dhaka University, Botanical garden.

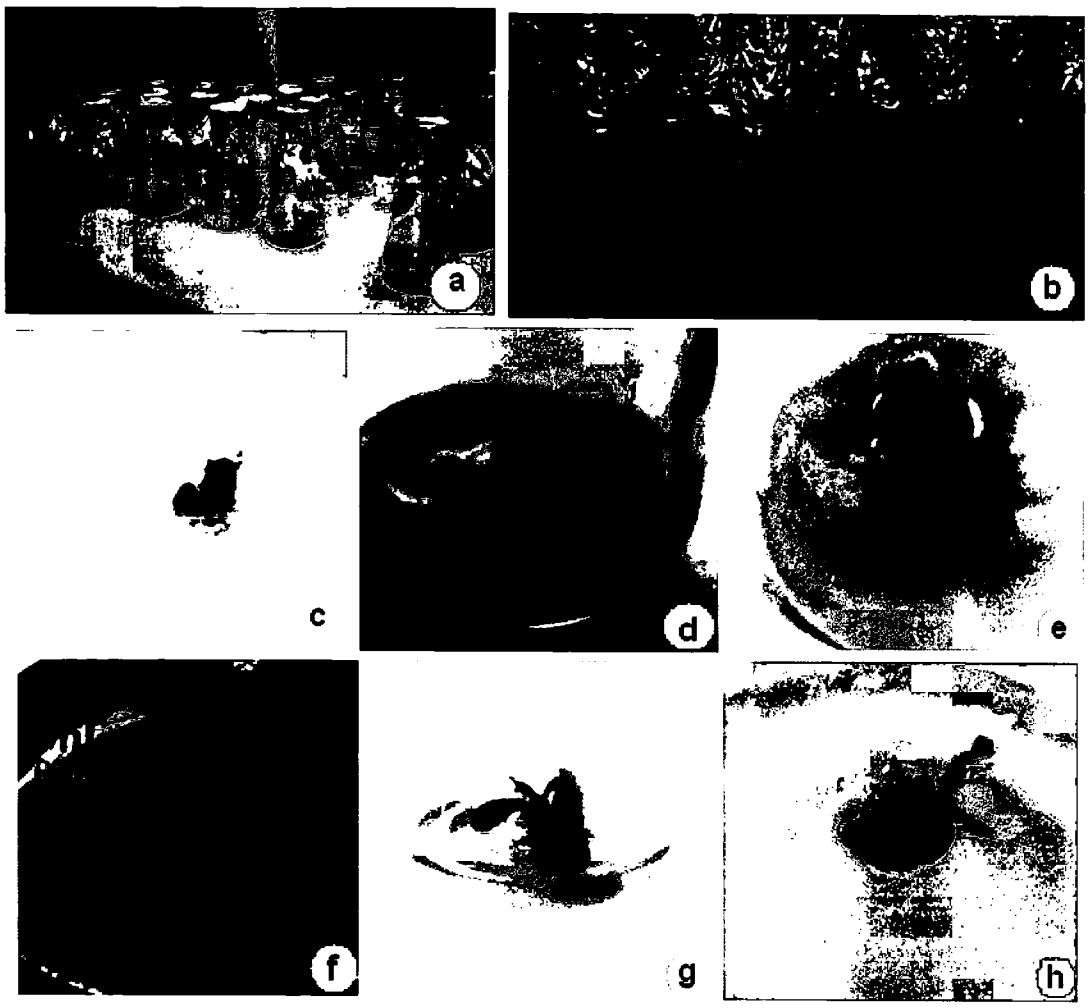

Plate: 3.2 *In vitro* culture and contamination of explants of Satkara (a) nodal explants inoculated in conical flask (b) nodal explants inoculated in test tubes (c) early contamination (d) (e) contamination after 14 -17 days (g) contamination after 21 -25 days & (h) contamination after sprouting.

B) Germination of seedlings and *in vitro* micrografting

Seed germination and seedling growth

The germination and growth of pummelo rootstock seedlings are presented in Table 3.2. Thirty number of pummelo seeds after decoating and surface sterilization cultured in MS basal media (MS_O) during October 2010. There were no germination of seeds after 7 days, but after 15 days 40% of the seeds germinated and seedling height was 0.75cm with two cotyledons. After 20 days only 74% seeds were germinated and seedling height was 2.35cm. At the age of 20 days, very young two leaves grown at the top and cotyledonary leaves were more expanded. No new seeds were germinated after 25 days. The seedling height increased 3.20cm at 25 days and 4.27 cm at 30 days. Within 25 - 30 days the seedlings were decapitated for micrografting.

In vitro micrografting

The performance of *in vitro* micrografted rootstocks are presented in Table: 3.3. Number of *in vitro* grown pummelo rootstocks and Satkara shoots were limited. 3 – 4 mm size buds were grafted on 4 - 4.5 cm long decapitated pummelo shoot. After 7 days of grafting only 20% buds were joined apparently but no contamination shown. The micrografted fresh, joined and contaminated shoots are presented in (Plate 3.3 j). The 40% of plants were contaminated after 10 days and at the advance stage the buds became yellowish and bluish spots shown at the joint. The 50% of the buds were dropped after 10 days. Finally, all joined grafts were contaminated and 60% of the buds dropped. At the end, within 20 - 25 days all buds along with stocks became dried (plate 3.3 k).

Table: 3.2 *In vitro* germination and growth of pummelo seedlings.

Days after Inoculation of seeds	Seeds germination (%)	Seedling length (cm)	No. of leaves	comments
07	00 (00%)	0.00 ± 0.00	00	No germination
15	12 (40%)	0.75 ± 0.28	02	Two cotyledonary leaves
20	21 (74%)	2.35 ± 0.20	04	Two very young leaves
25	21 (74%)	3.20 ± 0.15	04	Growth of leaves increase
30	21 (74%)	4.27 ± 0.23	04	Deep green leaves

Table: 3.3 Performance of *in vitro* micrografting of Satkara on Pummelo rootstocks.

Days of observation	No. of micro-grafted plants	No. of joined shoots	No. of contaminated grafts	No of dried buds	No of finally survived plants
04	10	00(00%)	00 (00%)	00 (00%)	00 (00%)
07	10	02(20%)	00(00%)	00 (00%)	00 (00%)
10	10	04(40%)	03(30%)	05(50%)	00 (00%)
15	10	04(40%)	04(40%)	06(60%)	No growth of buds
20	10	04(40%)	04(40%)	06(60%)	Bud & stock started to dry
25	10	04(40%)	04(40%)	06 (60%)	Buds with stock dried

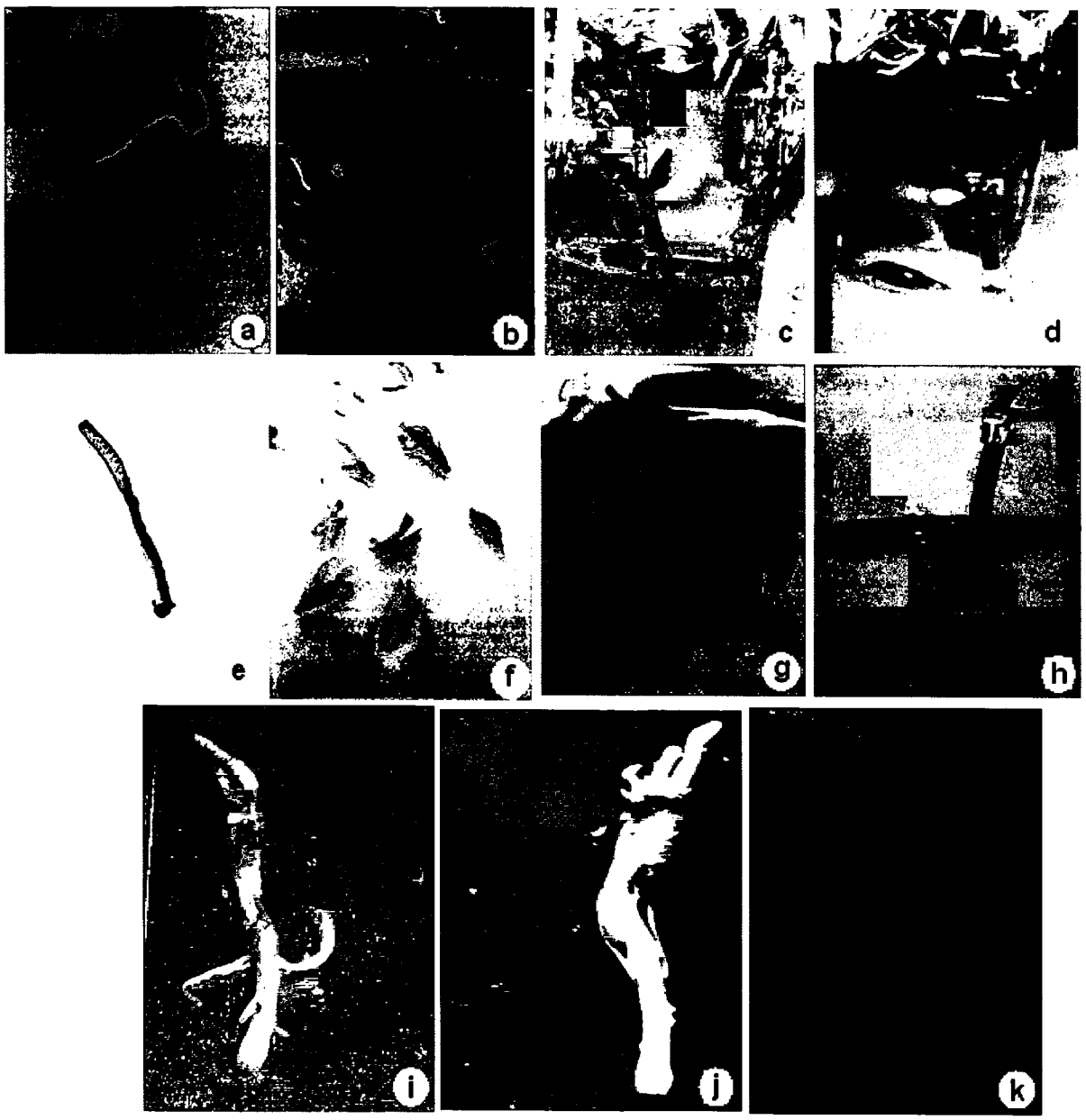

Plate: 3.3 *In vitro* germination of pummelo seedling and *In vitro* micrografting (a) & (b) germinating pummelo seeds (c) & (d) growth of seedlings (e) rootstock for grafting (f) buds for grafting (g) & (h) newly micrografted plants (i) bud joined with rootstock (j) contaminated grafted plants (k) dried and dead rootstock plants.

Ex vitro micrografting

Microshoots of 4 – 5 mm size with meristems collected from *in vitro* grown regenerated shoots. The shoot was placed on inverted T incised rootstock and bound with grafting tape. Then placed into plastic pot containing soil and compost (Coco dust) 1; 1 ratio.The results of 10 *ex vitro* micrografting plants are presented in Table 3.2.

Table: 3. 4 *Ex vitro* micrografting of Satkara bud on pummelo rootstock and their growth and survival rate.

Days of observation	No. of micro-grafted plants	No. of joined shoots	No. of dried shoots	Length of grafted plants (cm)	Comments
05	10	00(00%)	00 (00%)	3.5	Initial length
10	10	03(30%)	02(20%)	3.5	length remain same
15	10	03(30%)	05(50%)	3.5	length almost same
25	10	03(30%	07(70%)	3.5	bud start growth
35	10	03(30%)	07(70%)	4.2	single leaf
45	10	03(30%)	07(70%)	6.5	2 leaves
50	10	03(30%)	07(70%)	7.0	3 leaves
55	10	03(30%)	07(70%)	7.0	4 leaves
60	10	03(30%)	07(70%)	7.4	6 leaves

After 10 days of grafting, the 30% buds were joined with stocks and 20% buds became dried and dropped. After 25 days, the total 30% buds joined with stocks and 70% buds became dried and dropped. At this stage, the joined bud (single leaf) started to growth. The different stages of micrografting are presented in (Plate 3.4 a – h). After 15 - 25 days no new bud was joined and remaining 70% were dried and dropped. Length of grafted plant with single leaf became 4.2 cm at 35 days, 6.5 cm with two leaves at 45 days, 7.0 cm with three leaves at 50 days, 7.0 cm with four leaves at 55 days. The was length almost same (7.4 cm) after sixty days.

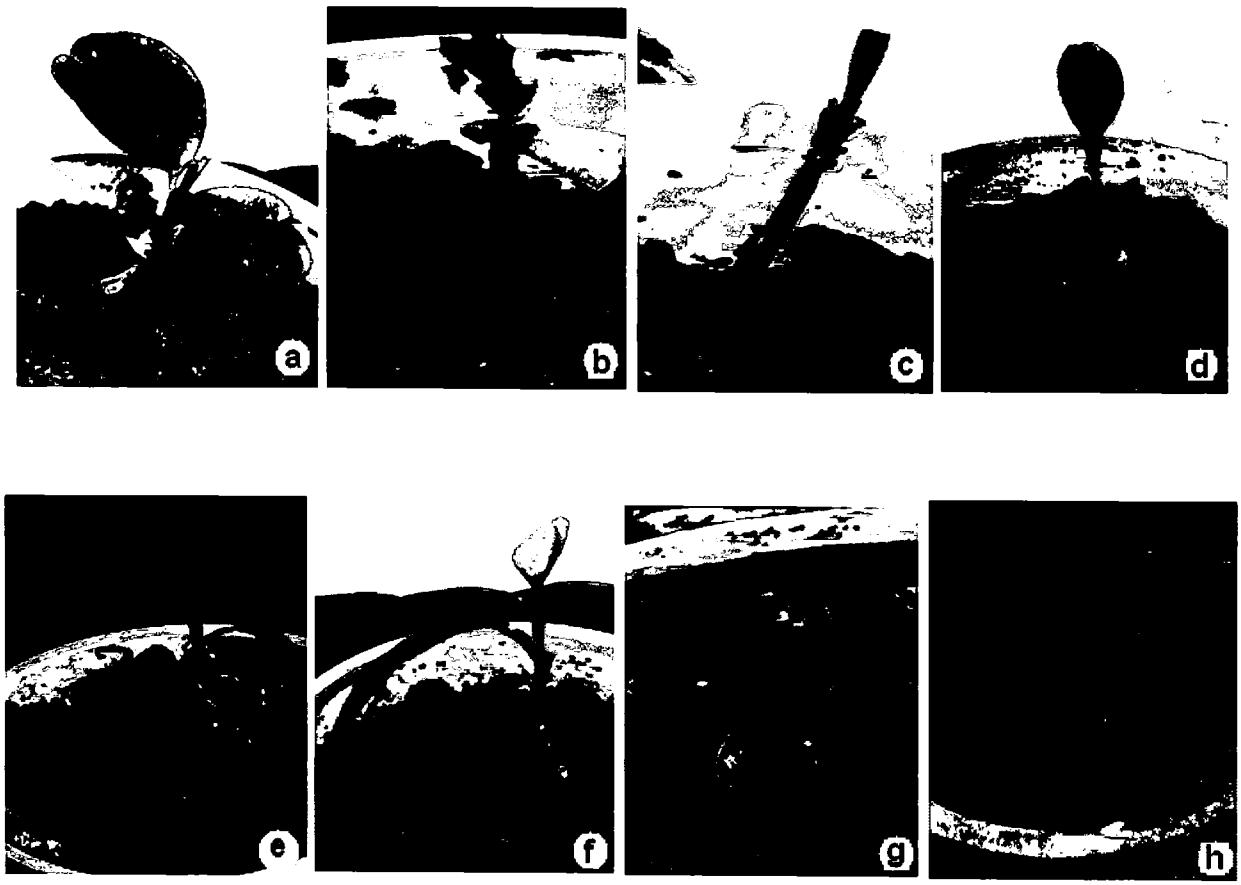

Plate: 3.4 *Ex vitro* micrografting of Satkara germplasm (a) Incised T grafting below cotyledonary leaves (b) and (c) open incised grafting (d) growth of newly joined bud (e) Two leaves developed; (f) Three leaves grafted plant (g) four leaves plant(h) Six leaves plant.

D) *In Vitro* shoot regeneration

In this experiment, expiants were collected from mature grafted plants grown in pots at Botanical garden of Department of Botany, University of Dhaka. From previous experiences and due to limited number of explants, BAP used as only growth regulator at three concentrations, 0.5 mg/l, 1.0 mg/l and 1.5 mg/l for shoot regeneration. The results are presented in Table: 3.5.

Table: 3.5 Effects of different concentrations of BAP supplemented MS medium on direct shoot regeneration from nodal explants of mature field plants of Satkara. Data (X ±. S.E) were taken after 5 weeks of culture.

Concentrations of BAP (mg/l)	No. of explants inoculated	Days to Shoot induction	% of explants responded	No. of shoots per explant	No of usable shoots per explant	Average length of shoot (mm)
0.5	10	7-9	25.00	1.25 ±0.20	0.75 ± 0.32	13.50±0.25
1.0	20	6-10	85.54	3.55 ±0.25	2.65 ± 0.24	18.30±0.20
1.5	10	8-10	40.25	1.65 ±0.26	1.20 ± 0.22	14.25±0.15

Shoot induction (Plate 3.5 a) was started after 6 days of inoculation and maximum induction done within 10 days. The highest percentage of shoot induction was (85.54%) recorded in BAP1.0 mg/l followed by 1.5 mg/l (40.25%) and 0.5 mg/l (25.00%). The highest number of microshoots also recorded in 1.0 mg/l supplemented medium (Plate :3. 5 e, f). Number of regenerated shoots and usable shoots were recorded highest (Shoots = 3.55 + usable shoots = 2.65) in 1 mg/l supplemented medium followed by 1.5 mg/l (Shoots = 1.65 + usable shots = 1.20). Average length of microshoots also highest (18.30mm) in 1.0mg/l supplemented medium followed by 1.5 mg/l (14.25) and 0.5 mg/l (13.5 mm)

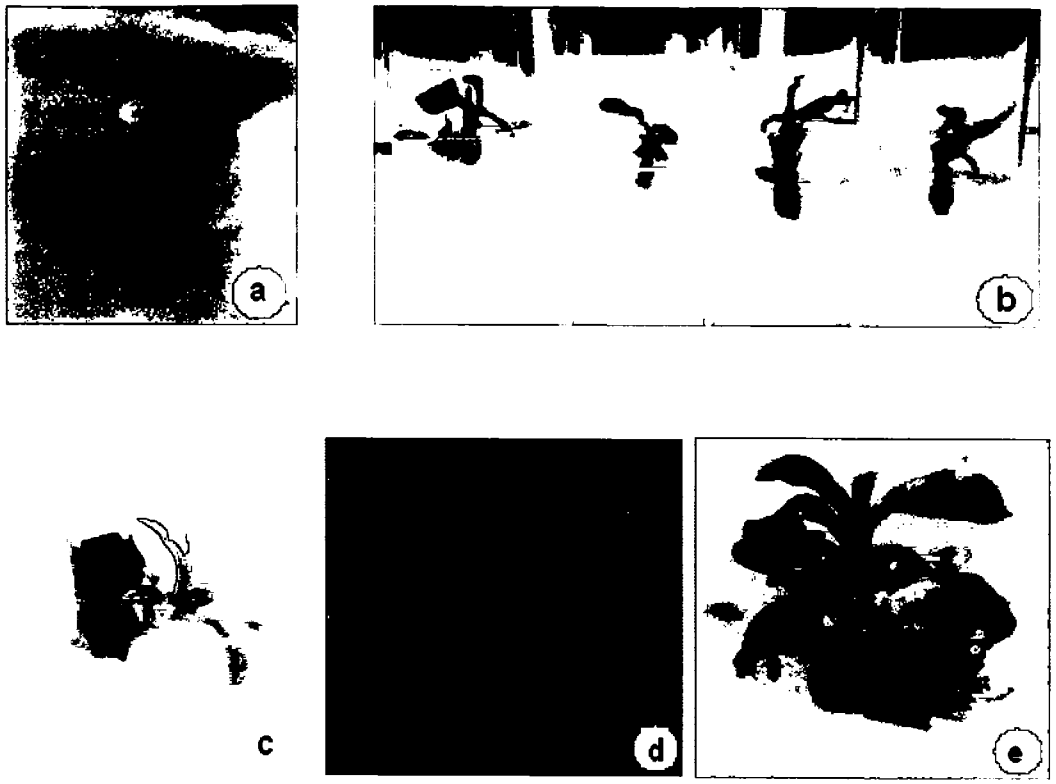

Plate: 3.5 Regeneration of shoots of Satkara germplasm (a) shoot induction (b) regeneration of shoots at 0.5 mg/lBAP (c - e) regeneration of shoots at 1.0mg/l BAP (d) contamination at later.

Subculture and transplantation of microshoots

Microshoots originated from BAP supplemented medium were sub cultured into same medium after 5 weeks. The results were not satisfactory. Some of the shoots elongated vertically without new branching and few were not elongated.

The microshoots of 16 -18 mm long with 3 - 4 leaves were directly transferred to the sterilized moist pot soil. Initially the plants were grown under growth room condition, then after 3 - 4 days plantlets with poly bags transferred to hardening room. Over 80% of inoculated shoots were survived (Plate 3.6 d & e).

Chapter - 11

Discussion

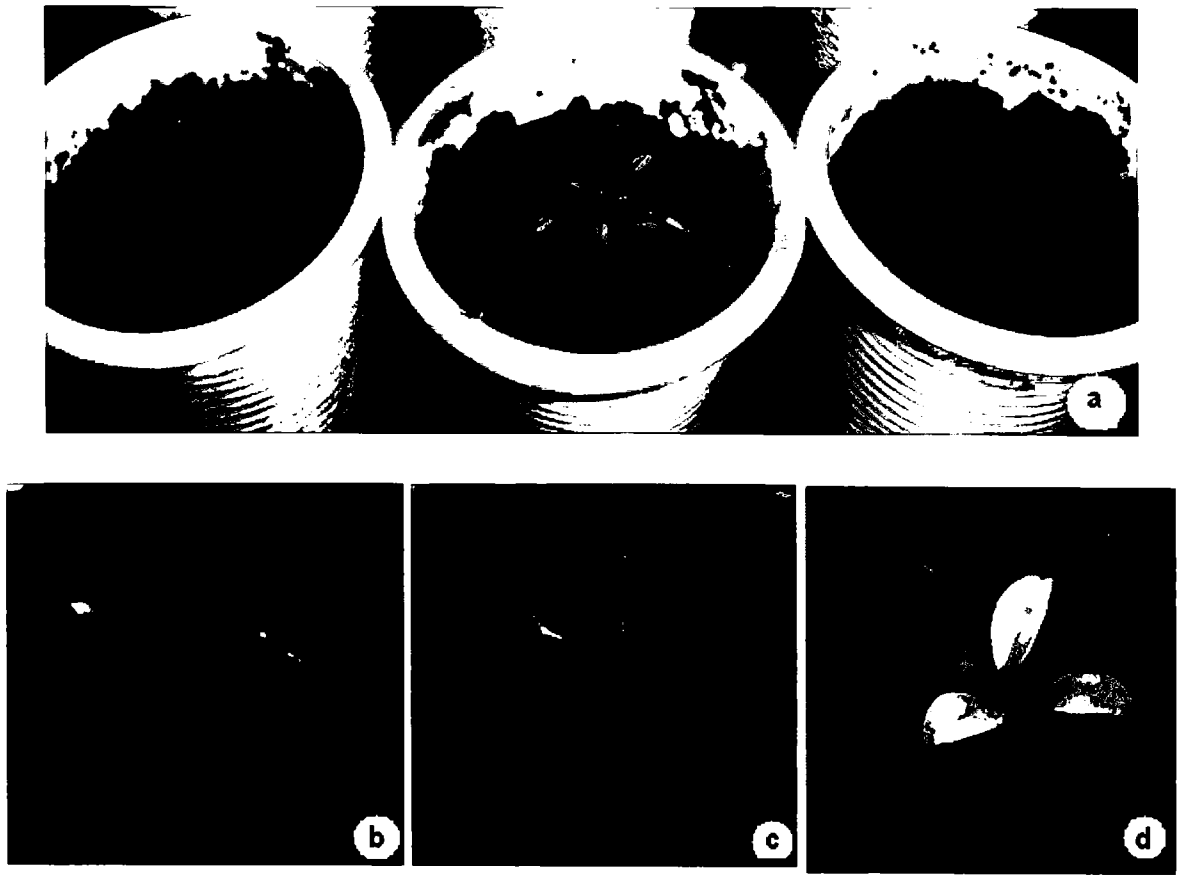

Plate: 3.6 Establishment of plantlets of Satkara (a) regenerated shoots established in pot soil (b) new leaf growth started (c) & (d) established shoots.

Discussion

Surface sterilization

The field grown tree buds are not easy to multiply in *in vitro* culture because they have high rate of contamination. The harsh tissue sterilization not only damages the growing regions of the buds but also effects their overall growth potential (Altaf, 2006). One of the familiar methods of *in vitro* propagation is the regeneration or sprouting of plantlets from nodal explants of citrus plants cultured on MS media supplemented with different concentration of BAP. Most of the works were done in citrus in *'in vitro'* grown seedling explants. The success rate was also satisfactory (Bonzid, 1975 in sweet and sour orange: Sim *et al.*, 1989 in *Citrus mitis*. Amin & Aktar,1993 in pummelo. Begum *et al.,* 2001 in pummelo). Practically it is very difficult to sterilize the field grown tree plants and sprouting of new shoots (Samarina *et al.,* 2010).

In vitro contamination by fungi, bacteria, or yeast is one of the most serious problems of plant tissue culture Laboratories (Leifert *et al.*, 1994). The establishment of an *in vitro* culture requires the removal of culturable fungal and bacterial contaminants. Contamination can be specially troublesome from field obtained materials (Bausher & Niedz, 1998). Several workers tried to regenerate shoots from field mature tree plants and became successful partially (Marin & Duran-vila, 1991 in *Citrus sinensis*. Altaf, 2006 in Kinnow. Miah *et al.,* 2008 in *C. macroptera*. Sen & Dewan, 2010 in Citronelo).

Altaf, (2006) obtained 31% clean explants by using 0.1%$HgCl_2$ for 10 minutes and 33% from 5% $Na(OCl)_2$ for 15 minutes in Kinnow mandarin tree. In present investigation the success rate of single chemicals was nil, but using of two chemicals one after another for 10 minutes each the success rate was 80%. It should be mentioned here that, the explants collected from Sylhet and after 20 - 22 hrs interval the materials were not responded well and contamination rate was also high.

Germination and *in vitro* micrografting

The germination ability of seeds in *in vitro* conditions varies from species to species. The hard seed coats of pummelo seeds prevent the moisture entrance and delayed or stopped the germination. So, before sterilization the hard seed coats were removed. Removal of the seed coat has been found to promote germination in Trifoliate orange (Singh and Soule, 1963 Okazaki *et al.,* 1964) and Troyer Citrange, (Muller and Welgemoed, 1980). Similarly, Amin and Akhtar, (1993) *Citrus grandis* (Pummelo), Miah *et al.,* (2008) in *C. macroptera* Mont.Var.*annamensis* in their experiments also removed hard seed coats before *in vitro* germination and obtained 100% success.

In present study, the rootstock of pummelo seedlings were grown *in vitro* and after 4 - 5 weeks when length of seedlings attained above 4cm then decapitated and buds (scion) from regenerated shoot were attached. The whole plant gown on BAP 0.5 mg/l + NAA 0.5 mg/l supplemented MS media. After 10 days 40% of the buds successfully joined but, later all the buds at joint were contaminated severely and became dried. *In vitro* shoot tip grafting were done by Juarej *et al.,* (1990) in *Citrus medica* L. and out of 51 attempts, 25 successful grafts were obtained. Only 16 plants survived after transplanting.

Two leaves primordial shoot tip of Washington navel orange grafted on Troyer citrange (3 - 5 cm length) and Citronello seedlings by two methods, inverted T-budding and contact with the vascular ring (Fifaei *et al.*, 2007). Sharma *et al.*, (2007) on Kinnow shoot tips of 0.7 mm size were excised from the sprouts of nodal segments and grafted on to rough lemon (*Citrus jambhiri* L) under aseptic condition. Aazami and Pouraghdam, (2010) were studied shoot-tip micro-grafting of some Iranian grapevine cultivars under *in vitro* conditions. Shoot tip explants of four cultivars were grafted on *in vitro* derived rootstocks of 4IB cultivar

Ex vitro micrografting

At present experiment, the pummelo rootstock seedlings were grown *ex vitro*, and buds collected from *in vitro* grown shoots. The bud was placed in an inverted -T incision on the rootstock and found good result. 30% of the buds joined successfully and growth was well. Edriss and Burger, (1984) in Mexican' lime, 'Valencia' orange and 'Star Ruby' grapefruit, the scion shoot tip was placed in an inverted T incision on the epicotyls of the rootstock (hybrid citrus) and found best result. Juarej *et al.*, (1990) in *Citrus medica* were obtained 49% successful grafts .

Microshoot grafting technique was successfully used in citrus plants by several workers (Kumarin & Singh, 2000 in rough lemon. Fifaei *et al.*, 2007 in naval orange). Therefore, the production of disease free foundation plants by micrografting remaining the only mean to supply disease free bud sticks to the growers (DeLang, 1978).

In vitro direct shoot regeneration from mature plant

One of the important objectives of plant tissue culture is to maintain the existing elite cultivars through *in vitro* culture from vegetative explants of mature plants. Successful protocols were developed by many researcher from different mature fruit trees, Jackfruit (Roy *et al.*, 1990, Amin and Jaisal 1993, Khanam and Bhuiyan 2001), Guava (Khatak *et al.*, 2001) and native olive (Rahman *et al.*, 2001). However, limited research has been carried on the regeneration of shoots and establishment of plantlets from mature citrus species and obtained few successes from *C. mitis* Blanco (Sim *et al.*,1989), *C. grandis* (Begum *et al.*, 2001), *C. macroptera* (Miah *et al.*, 2008) and lemon cultivars (Perez *et al.*, 2010).

For *in vitro* regeneration of shoots from nodal explants, three concentrations of BAP were used singly. Shoot induction was started after 6 days of inoculation and maximum induction done within 10 days. In all parameters like shoot induction.

Number of microshoots per explant, number of usable shoots per explant and length of shoots etc were best in BAP 1 mg/l supplemented medium.

Microshoots originated from BAP supplemented medium were sub cultured into same concentration medium. But after 5 weeks the results were not satisfactory. The microshoots of 16 -18 mm long with 3 - 4 leaves were directly transferred to the sterilized moist pot soil. Initially the plants were grown under growth room condition, then after 3 - 4 days plantlets with poly bags transferred to hardening room. The survival rates of the microshoots were 80%.

The *in vitro* rooting of the plantlets was very poor found from previous experiences (Miah *et al.*, 2008 in *C. macroptera*) and different reports. The microshoots directly transferred into pot soil for *ex vitro* rooting. The survival rate of the transferred shoots was also high (80%). The induction of *ex vitro* rooting from cultured shoots was more economical besides producing better and strong roots. *Ex vitro* rooting of shoots simultaneously combined with hardening of plantlets is thought to be better in terms of plant survival outside of culture than *in vitro* rooting. The main advantage of *ex vitro* over *in vitro* rooting is that root damage during transfer to soil is less. The rooting rate is higher and root quality is often better when the rooting takes place *ex vitro.*

The present results were vary in some cases and closed to other citrus species in most cases. The development of axiliary buds depended largely on the age of the plant (explants source) and the variety, (Shengeliya and Butenko ,1986).

Chapter - 12

Conclusion

Conclusion

The *Citrus macroptera* are generally propagated through bud grafting. Propagation is therefore, limited to the period when buds are available. These conventional techniques are also not free from risk of inborn pathogens. However, *in vitro* micropropagation, microshoot grafting techniques can overcome the some constraints to citrus improvement and cultivation and can increase fruit quality and resistance to diseases and environmental stresses.

The first and foremost step of micropropagation for mature field explants is surface sterilization. The field grown tree buds are not easy to multiply in culture because they have high rate of contaminations in *in vitro* growth. The harsh tissue sterilization not only damages the growing regions of the buds but also affect their overall growth potential. In case of sterilization, explants were treated immediately after collection. Because of long gap the growth capacity of stem tissues reduced and population of inborn microbes increased. For surface sterilization, the uses of both sterilizing chemicals (5% Na $(OCl)_2$ for 10 minutes + 0.1% $HgCl_2$ solution for 10 minutes) were found most effective IN Satkara.

For *in vitro* micrografting, the pummelo seedlings were used as rootstock and grown in *'in vitro'* condition The seedling length became 4.27 cm at 30 days. Within 25 - 30 days the seedlings were decapitated for micrografting. 3 – 4 mm size buds were joined on 4 - 4.5 cm long decapitated pummelo shoot. The 50% of the buds were dropped after 10 days and 50% became successful. Finally, all joined grafts were contaminated and within 20 - 25 days all buds along with stocks became dried and dead

In case of *ex vitro* grafting, buds from *in vitro* grown shoot were grafted with *ex vitro* grown pummelo seedlings. The total 30% of buds joined with stocks and 70% of buds became dried and dropped. After 15 - 25 days no new bud was joined and remaining were dried and dropped. Single leaf with grafted plant height became 4.2cm at 35 days, 6.5 cm with two leaves at 45 days, 7 cm with three leaves at 50 days, 7 cm with four leaves at 55 days. The length almost same (7.4 cm) after sixty days.

For *in vitro* regeneration of shoots from nodal explants, three concentrations of BAP were used singly. Shoot induction was started after 6 days of inoculation and maximum induction done within 10 days. The highest percentage of shoot induction was (85.54%) recorded in 1 mg/l followed by 1.5 mg/l (40.25%) and 0.5 mg/l (25.00%). The highest number of microshoots also recorded in 1 mg/l medium. Number of regenerated shoots and usable shoots were recorded highest in 1mg/l supplemented medium. Average length of microshoots was also highest (18.3 mm) in 1mg/l supplemented medium.

Microshoots originated from BAP supplemented medium were sub cultured into same condition after 5 weeks. The results were not satisfactory. The microshoots of 16 -18 mm long with 3 - 4 leaves were directly transferred to the sterilized moist pot soil. Initially the plants were grown under growth room condition, then after 3 - 4 days plantlets with poly bags transferred to hardening room. The survival rates of the microshoots were 80%.

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APPENDICES

Appendix - 1

Murashige and Skoog (MS) Medium,1962

Constituents	Concentrations (mg/l)
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2,2H_2O$	440.00
$MgSO4,7H_2O$	370.00
Micronutrients	
$FeSO_4,7H_2O$	27.80
Na_2EDTA	37.30
$MnSO_4, 4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4, 7H_2O$	8.60
KI	0.83
$Na_2Mo_4, 2H_2O$	0.25
$CuSO_4, 5H_2O$	0.025
$CoCl_2, 6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Ionositol	100.00
Sucrose	30,000.00
Agar Powder	85,00.00

pH was adjusted to 5.7± before autoclaving and Agar mix

Appendix- 2

The Rainfall and temperature data of Sylhet during, 2008.

Month name (2008)	Rainfall (**mm**)	Temperature(^{0}C)	
		Highest	Lowest
January	20.00	24.40	14.40
February	35.20	26.20	14.00
March	187.70	29.90	19.20
April	137.60	33.10	22.50
May	588.90	32.60	23.50
June	573.10	31.80	24.80
July	594.40	32.00	25.50
August	760.30	31.50	25.20
September	257.20	32.80	25.10
October	192.80	31.60	23.00
November	00.00	29.90	18.00
December	00.00	27.40	16.50
Average	334.72mm	30.59^{0}C	21.00^{0}C

Source: Abhawa Office, Sylhet

Appendix - 3

The Rainfall and temperature data of Sylhet during, 2009.

Month name (2009)	Rainfall (mm)	Temperature(^{0}C)	
		Highest	Lowest
January	Trace	26.60	14.40
February	20.40	30.50	15.70
March	101.30	32.60	19.27
April	419.70	32.50	22.80
May	553.80	32.50	23.30
June	511.80	32.20	24.90
July	580.30	33.00	26.10
August	712.50	32.10	25.60
September	251.10	32.70	25.50
October	136.30	32.70	23.20
November	Trace	30.00	19.60
December	00.00	27.10	14.80
Average	328.72mm	31.79^{0}C	22.07^{0}C

Source: Abhawa Office, Sylhet.

Appendix - 4

The Rainfall and temperature data of Sylhet during, 2010.

Month name (2010)	Rainfall (mm)	Temperature(^{0}C)	
		Highest	Lowest
January	00.00	30.8	10.00
February	00.50	32.00	11.60
March	221.50	37.10	17.40
April	733.10	35.00	17.50
May	749.90	35.00	19.40
June	921.70	34.90	21.70
July	558.00	35.70	24.70
August	735.70	35.90	24.50
September	732.20	35.50	23.00
October	231.10	36.00	20.00
November	20.70	33.60	16.50
December	45.50	30.80	12.40
Average	412.50mm	34.79^{0}C	19.97^{0}C

Source: **Abhawa Office, Sylhet.**

Appendix – 5

Import statistics of Satkara from India through different borders of Sylhet division.

SL Nos.	Financial year	Metric tons(MT)
1.	2008 – 2009	250.128
2.	2009 – 2010	215.051
3.	2010 – 2011	185.756

Source: Customs Department, Sylhet, 28/7/2011

ANNEXURE

ISSN 0972-060X

Composition of the Volatiles of *Citrus macroptera* var. *annamensis* and Evaluation of Bioactivity

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Received 31 July 2009; accepted in revised form 17 December 2009

Abstract: The distillation and subsequent extraction of the distillate obtained from the fresh peels of the fruits of *Citrus macroptera* var. *annamensis* afforded 120 mg of oil (yield 0.12 %). Analyses both by gas chromatography (GC) and gas chromatography- mass spectrometry (GC-MS) of the sample resulted in the identification of 25 terpenoids, predominantly monoterpene hydrocarbons, accounting for 97.0 % of the total oil, and limonene (73.5 %) as the major component. While the oil did not exhibit any *in vitro* free-radical-scavenging (DPPH), it showed considerable antibacterial activity against *Bacillus cereus, B. subtilis*, *Escherichia coli* and *Staphylococcus aureus* with the MIC values ranging from 1.25 to 5.0 mg/mL.

Key words: *Citrus macroptera* var. *annamensis,* Rutaceae, volatile oil, GC and GC-MS analysis, limonene, bioactivity

Introduction: *Citrus macroptera* Mont. var. *annamensis* Tanaka, commonly known as 'Satkara', is an important citrus fruit crop of the family Rutaceae.[1] The average height of this plant is 2-3 m and width of 2-4 m. The fruit of this species is edible and popular

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among the people of greater Sylhet region of Bangladesh, and Meghalaya and Assam of India. In Bangladesh, both green and mature fruits are used in cooking for flavouring curry, and also for pickle preparation. The fruits are used as a traditional medicine by the local tribes of Assam in India [2]. Chowdhury *et al.*[3] have recently reported the antioxidant activity of the crude extract of this plant. While there are no reports available to date on any phytochemical studies on this particular variety of *C. macroptera*, a few previous studies on other varieties of *C. macroptera* revealed the presence of a quinolone alkaloid and coumarins [4-6].

As part of our on-going studies on Bangladeshi medicinal plants [7-20], we now report on the composition of the distilled peel volatile oil of the fruits of *Citrus macroptera* Mont. var. *annamensis* and evaluation of its bioactivity.

Experimental

Plant material: Fresh mature fruits of *Citrus macroptera* Mont. var. *annamensis* Tanaka were collected in November 2008 from the Citrus Research Station, Bangladesh Agricultural Research Institute (BARI), Jaintapur, Sylhet, Bangladesh. A voucher specimen was submitted to the National Herbarium Bangladesh and identified with the accession number - DACB 34217. After harvesting, the fruits were wrapped with clean poly bag and preserved in the refrigerator until extraction.

Volatile oil extraction: Fresh peels (flavedo and albedo, 100 g) from the fruits were cut into small pieces by clean sharp knife. The peels were placed in a 1 L round bottom flask. Distilled water (600 mL) was added to facilitate distillation process. The flask was fitted with distillation apparatus. The distillate was collected in a flask. The distillation was continued for 4 h. The distilled portion was extracted three times with 50 mL *n*-hexane immediately after distillation. The *n*-hexane part was dried over anhydrous sodium sulphate. The extract was evaporated to dryness in a vacuum evaporator to obtain the volatile oil.

GC-MS analysis: The GC-MS analysis was performed in an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as a carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to rise 220°C at a rate of 4°C /min, and kept constant at 220°C for 10 min. and then programmed to rise to 240°C at a rate of 1°C/min. Split ratio was calibrated at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

GC analysis: The GC analysis was carried out using an Agilent 6890N GC system. The FID detector temperature was 300°C. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results are expressed as mean percentage ± standard deviation (SD) (n = 3) (Table 1).

Identification of components: Identification of the volatile oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Identification of compounds was based on computer matching using the Wiley GC-MS Library, Adams Library and MassFinder 3 Library [21,22], and comparison of the MS data with literature data [23,24].

Bioactivity

Quaiitative 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay: DPPH, molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. The free-radical-scavenging effect of the volatile oil (5 mg/mL) was assessed using the qualitative DPPH [25,26]. DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 mg/mL. Test samples were applied on a Silica gel pre-coated TLC plate (0.25 mm thickness), air-dried and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were noted. Trolox® (1 mg/mL), purchased from Sigma-Aldrich, UK, was used as a positive control.

Antibacterial assay incorporating resazurin as an indicator of cell growth: The antibacterial activity of the volatile oil was assessed against six bacterial strains, *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (NCTC 10400), *Escherichia coli* (ATCC 8739), Ampicillin-resistant *Escherichia coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 1803) and *Salmonella typhii* (NCTC 10203). The recently published 96-well microtitre assay using resazurin as the indicator of cell growth [27] was employed for the determination of the minimum inhibitory concentration (MIC) of the volatile oil. Ciprofloxacin (purchased from Fluka Chemie AG, Bucks), a broad-spectrum antibiotic, was used as a positive control. The bacteriostatic bactericidal property of the volatile oil was carried out as follows: agar plate was seeded with the mixture from the well which was just before the well of the MIC, using sterile transfer loop and kept at 4°C to facilitate maximum diffusion. The plates were kept in an incubator (37°C) to allow the growth of the bacteria. Any bacterial growth would indicate the bacteriostatic property of the extract, and no growth would be an indicator of bactericidal activity.

Results and discussion: The distillation of fresh peels of the fruits of *Citrus macroptera* var. *annamensis* produced 120 mg of volatile oil (yield 0.12 %). The GC-MS analyses of the volatile oil led to the identification of 25 compounds accounting for over 97.0 % of the total oil (Table 1). The majority of components present in the oil were monoterpenes. There were 15 monoterpenes, six of which possessed cyclic structure, e.g., limonene, 1,8-cineole, α-copaene, γ-terpinene, terpinen-4-ol and α-terpineol, and nine others had straight chain skeleton. Six sesquiterpenes, e.g. β-caryophyllene, α-humulene, δ-cadinene, elemol, γ-eudesmol and nootkatone were among 25 identified compounds. There were four nonterpenoidal alkane derivatives, e.g. octanal, tridecane, nonanal and octanol. Among the identified components, limonene was the major compound contributing about 74.0 % of the total oil. This finding was consistent with the previous studies on other

Citrus species where limonene was reported as the major compound in the *Citrus* volatile oils [28-31]. 1,8-Cineole, α-humulene, geranial, geraniol and nerol were identified in trace amounts. While α-terpineol and δ-cadinene were present within the range of 3-4 %, β-caryophyllene, γ-eudesmol and nootkatone contributed about 2 % each to the composition.

The DPPH antioxidant assay is based on the principle that 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, can be decolourised in the presence of free-radical-scavengers. The odd electron in the DPPH radical is responsible for the absorbance at 517 nm, and also for visible deep purple colour [26]. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolourised. In the TLC-based qualitative free-radical-scavenging assay using the DPPH spray the volatile oil did not show any free-radical-scavenging property, which was evident from any white/yellowish-white spots against the pink background of the TLC plate sprayed the DPPH spray.

The minimum inhibitory concentrations (MIC) of the volatile oil of *Citrus macroptera* var. *annamensis* against six bacterial strains were determined by the recently developed resazurin microtitre assay [27]. The volatile oil displayed reasonable antibacterial activity against four of the test strains including, *Bacillus cereus*, *B. subtilis*, *Escherichia coli* and *Staphylococcus aureus*, but not against ampicillin-resistant *E. coli* and *Salmonella typhii* (Table 2). The most potent activity was observed against both *Bacillus* species with an MIC value of 1.25 mg/mL. The antibacterial activity of the volatile oil was found to be bacteriostatic. The antibacterial activity profile displayed by the volatile oil of *Citrus macroptera* var. *annamensis* was similar to that observed with *Citrus sinensis* volatile oil [30].

To the best of our knowledge this is the first GC and GC-MS study on the volatiles of *C. macroptera* var. *annamensis*, as well as the *in vitro* evaluation of its antibacterial and free-radical-scavenging activities.

Acknowledgements: One of us (MNM) is grateful to the Uiversity Grants Commision (UGC) of Bangladesh for providing a research fellowship. Authors are thankful to the Bangladsh Agriculture Research Institute (BARI), Jaintapur, Sylhet, for their active support.

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Table 1. Composition of the volatile oil of *Citrus macroptera* Mont. var. *annamensis* Tanaka

Compound	RRI[a]	%
Myrcene	1174	0.67 ± 0.12[b]
Limonene	1203	73.53 ± 2.54
1,8-Cineole	1213	tr
γ-Terpinene	1255	0.37 ± 0.06
Octanal	1296	0.40 ± 0.10
Tridecane	1300	0.47 ± 0.12
Nonanal	1400	1.63 ± 0.06
trans-Linalool oxide (furanoid)	1450	0.67 ± 0.15
cis-Linalool oxide (furanoid)	1478	0.43 ± 0.06
α-Copaene	1497	1.60 ± 0.10
Linalool	1553	1.17 ± 0.06
Octanol	1562	0.57 ± 0.06
Terpinen-4-ol	1611	0.30 ± 0.17
β-Caryophyllene	1612	1.97 ± 0.32
α-Humulene	1687	tr
Neral	1694	0.53 ± 0.06
α-Terpineol	1706	3.00 ± 0.27
Geranial	1740	tr
δ-Cadinene	1773	3.43 ± 0.15
Nerol	1808	tr
Geraniol	1857	tr
(*E*)-Nerolidol	2050	1.43 ± 0.12
Elemol	2096	0.57 ± 0.21
γ-Eudesmol	2185	2.13 ± 0.21
Nootkatone	2554	2.13 ± 0.25
Total		97.0 ± 2.62

[a] RRI Relative retention indices calculated against *n*-alkanes on the HP Innowax column;
[b] mean % calculated from Flame Ionization Detector (FID) data ± SD (n= 3)
[c] tr, trace (< 0.1 %)

Table 2. Antibacterial activity of the volatile oil of *Citrus macroptera* var. *annamensis* (CMA)

Bacterial strains	Antibacterial activity MIC in mg/mL	
	CMA	Ciprofloxacin
Bacillus cereus (ATCC 11778)	1.25	2.5×10^{-7}
Bacillus subtilis (NCTC 10400)	1.25	2.5×10^{-7}
Escherichia coli (ATCC 8739)	2.5	2.5×10^{-7}
Ampicillin-resistant *Escherichia coli* (NCTC 10418)	No activity	2.5×10^{-6}
Staphylococcus aureus (NCTC 1803)	5.0	2.5×10^{-8}
Salmonella typhii(NCTC 10203)	No activity	2.5×10^{-7}