CHAPTER 4 RESULTS AND DISCUSSION

4.1 LITERATURE REVIEW:
An extensive search on the literature reviews about the plant was conducted. *Manilkara zapota* (L). P. Royen has been reported to possess various therapeutic utilities as antioxidant, anti-inflammatory, anthelmintic, antibacterial against various gram positive and gram negative organisms etc., however its sunscreen activity as well as anti-acne activity against acne causing organisms was unexplored. Also the nutraceutical potential of the fruit though proved, was not reported to be used in formulation development. Hence the present research aimed to overcome the lacunae in this area.

4.2 PROCUREMENT AND AUTHENTICATION OF MANILKARA ZAPOTA (L.) PLANT:
Fresh leaves, ripe fruit and bark of *Manilkara zapota* (L). P. Royen synonym *Achras zapota* L. plant commonly known as Chicle, Sapodilla, chiku of family Sapotaceae were collected from the local garden of the St. John Institute of Pharmacy and Research (St. John Technical Campus) District Palghar, Maharashtra, India.

Leaves of the plant were separated, washed thoroughly with tap water, wiped with clean cloth, shade dried, coarsely grinded and stored in air tight container. Bark of the plant was shade dried and coarsely grinded and stored in air tight container. The ripe fruits were collected and immediately studied. The plant was further authenticated and confirmed that the plant material belongs to *Manilkara zapota* (L.) P. Royen synonym Achras sapota L. commonly known as chicle, Sapodilla, Chiku of the family Sapotaceae, by Dr. Harshad M. Pandit, Head of the Department of Botany, Guru Nanak Khalsa College of Arts, Science and Commerce, Matunga, Mumbai and the specimen no. #nlr 080314 is deposited in the Herbarium of the Department of Botany, Guru Nanak Khalsa College, Matunga, Mumbai, Maharashtra, India. The authentication certificate for *M. zapota* plant is given in Chapter no. 5.1, Figure no. 5.1.4.2.1.

The leaves are spirally arranged simple elliptic or oblong apex obtuse to shortly acuminate coriaceous shining glabrous when mature. The fruit is ovoid to globular berry with a rough brown skin with 1-12 shining brown or black seeds surrounded by a
brownish sweet juicy scented soft tissue. The bark pieces are dark brown hard and deeply fissured forming small rectangular pieces.

4.3 STANDARDIZATION OF MANILKARA ZAPOTA (L.) PLANT:

DETERMINATION OF PROXIMATE VALUES:
The following proximate values were determined for the powder drug of the plant Manilkara zapota.

4.3.1 DETERMINATION OF EXTRACTIVE VALUE:
The procedure used to determine Extractive values basically helps to estimate the amount of soluble constituents present in a specified amount of the coarse material obtained from the medicinal plant, on extraction with solvents. Different phytoconstituents are thus present in the solution obtained on the extraction of the crude drug using a specific mentrum as a solvent. Specific solvent used are the important factors affecting the composition of these phytoconstituents in that particular solvent. The results of the alcohol soluble and water soluble extractive values estimated for the dried leaves and bark powder of Manilkara zapota are tabulated in Chapter no. 5.2, Table no. 5.2.4.3.0. The comparison of the data revealed that the extents of phytoconstituents soluble in alcohol are present to a lesser extent in leaves to that of the water in leaf powder. However the extents of phytoconstituents soluble in alcohol are more in bark powder to that of the water. This could also be useful in interpreting that the hydrophilic nature phytoconstituents are more in leaves powder than that of bark powder.

4.3.2 DETERMINATION OF ASH VALUES:
The determination of Ash values helped to determine the extent of adulterants like inorganic contaminants if any are present in the crude drug powder. The details of the total ash values, alcohol insoluble and water soluble ash values determined for the dried leaves and bark powder of Manilkara zapota are tabulated in Chapter no. 5.2, Table no. 5.2.4.3.0. The comparison of the data revealed that the extents of total ash and water soluble ash value are higher in bark powder to that of the leaf powder. However the acid
insoluble ash values are similar in both powders. This also indicates that the adulterants like inorganic contaminants are more in the bark powder than that of the leaves powder. Also since the bark obtained from the plant was not washed but directly shade dried hence chances of dust settling on the surface could be the possible reason for more extent of inorganic contaminants, while the leaves were thoroughly washed.

4.4 PRELIMINARY RESEARCH:

The preliminary research was conducted to determine whether the plant possesses any sunscreen activity so as to proceed further with plant. Hence successive extraction of coarsely powder material (leaves, bark and seed) using soxhlet extractor with different polarity solvents was carried out. The spectra of the crude extract thus obtained as shown in Chapter no. 5.1, Figure no. 5.1.4.4.1 was compared with each other. Based on the spectra it was further decided to select the hydroalcoholic (water-ethanol) (3:7) as a solvent for extraction. Also from the spectra comparison of the bark, leaves and seed, it was decided to proceed the further studies only with bark and leaves extract since seed extract gave a very low absorbance at the same concentrations.

Further the method of extraction to be used in the research work to get the desired extract was decided based on the comparison of the spectra measured using the extracts obtained by Soxhlet extraction and maceration technique as shown in Chapter no. 5.1, Figure no. 5.1.4.4.2. Both the spectra were identical; the soxhlet extract was found to reveal slight higher extraction efficiency however maceration process was much quicker compared to soxhlet extraction hence it was selected as the method of extraction.

4.5 PHYTOCHEMICAL INVESTIGATION:

4.5.1 EXTRACTION:

Maceration technique was employed in extraction of the M. zapota leaves and bark extract using hydroalcoholic solvent. The method in comparison to the soxhlet extraction technique was much faster and the extracts were also concentrated quickly. The dried extracts obtained by the maceration technique were free flowing and non-sticky in nature.
4.5.2 DETERMINATION OF EXTRACTIVE YIELD:

The coarse powder of *M. zapota* leaves and bark upon maceration with solvent, water : ethanol (3:7) yielded 26.5 % w/w dark green and 51.36 % w/w dark brown solid hydroalcoholic extracts respectively.

4.5.3 QUALITATIVE PHYTOCHEMICAL INVESTIGATION:
4.5.3.1 CHEMICAL TEST:

The qualitative chemical analysis was carried out to know the presence of different phytoconstituents in each extracts of *M. zapota* leaves and bark. The results thus obtained are tabulated in Chapter no. 5.2, Table no. 5.2.4.5.3.1.1. The phytoconstituents like phenol, flavonoids-flavones, glycosides, coumarins, alkaloids, tannins, terpenoids etc. were found to be present.

4.5.3.2 THIN LAYER CHROMATOGRAPHY:

Thin layer chromatographic (TLC) studies confirmed the presence of Flavonoids, coumarins, alkaloids and steroidal glycosides in different solvent systems employed. Although some solvent system revealed the absence of alkaloids, decreasing the polarity of the system showed a typical alkaloid spot in the chromatogram. The photographs of the TLC plates are given in Chapter no. 5.1, Figure no. 5.1.4.5.3.2.1.

4.6 PHYSICOCHEMICAL CHARACTERIZATION OF THE EXTRACTS:
4.6.1 DESCRIPTION OF EXTRACTS:

The physicochemical characteristics of the extracts were determined and tabulated in Chapter no 5.2, Table no. 5.2.4.6.1.1. The extracts were found to exhibit a dark green and
brown color for the leaves and bark extracts respectively with a characteristic odor. Also they were free flowing and non-sticky.

4.6.2 FT-IR SPECTRA:
FT-IR spectra of the hydroalcoholic extract of *M. zapota* leaves and bark extract revealed functional groups as tabulated in Chapter no 5.2, Table no. 5.2.4.6.2.1. The spectra thus obtained for the leaves and bark extracts are reported in Chapter no 5.1, Figure no. 5.1.4.6.2.1 and Figure no. 5.1.4.6.2.2 respectively. Based on the peaks observed, the phenolic like phytoconstituents having some polar functional groups and aromatic ring structures are confirmed present in the hydroalcoholic extracts of leaves and bark.

4.7 QUANTITATIVE PHYTOCHEMICAL ANALYSIS:
4.7.1 DETERMINATION OF TOTAL PHENOL CONTENT:
Principle:
The Folin-Ciocaiteau method also called as Gallic acid equivalent method was employed in determination of the phenol content of the test extracts. The Gallic acid equivalent method, uses a mixture of phosphomolybdate and phototungstate used for the colourimetric assay of phenolic antioxidants. The Folin-Ciocalteau assay method is based on the electron transfer. The reducing capacity is measured which is corresponding to the phenolic content of biological materials. The basic fact is that the phenols are found to ionize completely in alkaline conditions thereby oxidized readily further by using the Folin-Ciocalteau reagent. The reaction gives a blue coloured complex of phosphomolybdic/ phosphotungstic acid which can then be spectrophotometrically determined at 765 nm.

The test extracts revealed very good amount of phenols. The values obtained using the standard curve of Gallic acid (y = 0.013x+0.448) (Chapter no 5.1, Figure no. 5.1.4.7.1.1) were very close in both the extracts and computed to 330.77 ± 0.71 mg/g and 333.08 ± 0.96 mg/g respectively of gallic acid equivalent per gm of dry extracts as given in Chapter no 5.2, Table no.5.2.4.7.1.1. The results thus obtained for hydroaclcoholic *M. zapota* leaves extract is higher than that reported by Chanda S. *et al.*, 2012, for the
petroleum ether, toluene, ethyl acetate, acetone and aqueous *M. zapota* leaves extracts. Thus the phenolic constituents were found to be higher in the hydroalcoholic extracts of *M. zapota* leaves and bark.

4.7.2 DETERMINATION OF TOTAL FLAVONOID CONTENT:

**Principle:**

On addition of aluminium chloride, it reacts with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols to form complexes. These complexes are acid stable in nature. Aluminium chloride is found to form complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids which are acid labile. Quercetin was used as a standard reference in the procedure and a standard curve using different concentrations was plotted.

The test extracts revealed very good amount of flavonoids. The standard curve of Quercetin used was \( y = 0.018x - 0.014 \) (Chapter no 5.1, Figure no. 5.1.4.7.2.1). Comparatively hydroalcoholic *M. zapota* leaves extract constitute more flavonoids content computed to 183.89 ± 2.3 mg/g of Quercetin equivalent per gm of dry extract than that of the bark extract of 47.39 ± 1.2 mg/g of Quercetin equivalent per gm of dry extracts as given in Chapter no 5.2, Table no. 5.2.4.7.2.1. The results thus obtained for hydroalcoholic *M. zapota* leaves extract is higher than that reported by Chanda S. *et al.*, 2012, for the petroleum ether, toluene, ethyl acetate, acetone and aqueous *M. zapota* leaves extracts as well as methanolic *M. zapota* leaves extract reported by Hossain Hemayet *et al.*, 2012.

4.8 ANTIOXIDANT ACTIVITY OF THE EXTRACTS:

4.8.1 REACTION WITH 1,1-DIPHENYL-2-PICRYL HYDRAZYL (DPPH):

**Principle:**

DPPH molecule constitutes a free radical which is stable in nature. When an an antioxidant able to donate an electron to DPPH is present in the vicinity, the typical purple color of the free DPPH radical is found to be decayed. This leads to the change in absorbance which is measured spectrophotometrically at 517 nm. Based on this simple
test an information regarding the capability of a compound in donating a hydrogen atom, also the number of electrons a given molecule is able to donate, and the mechanism of antioxidant action can be obtained. In cases of plant extracts wherein the chemical structure of the electron donor is unknown, this method also helps to provide information regarding the reduction potential of the sample, and hence it could be useful to compare the reduction potential exhibited by the unknown materials.

Mechanism:

The *Manilkara zapota* extracts were thus screened for the free radical scavenging potentials using 1,1-diphenyl-2-picryl hydrazyl (DPPH) dissolved in methanol. Antioxidants basically react with the DPPH molecule thereby converting it to 1,1-diphenyl-2-picryl hydrazine. A discoloration in color is thus seen. The degree of discoloration produced further indicates the scavenging potential of the extract comprising the antioxidants.

Thus the free radical scavenging potential of the hydroalcoholic *M. zapota* leaves and bark extracts were tested against a methanolic solution of DPPH. Leaves extract exhibited a lower IC$_{50}$ computed to 61.2 ± 0.002 μg/ml as compared to the bark extract of 85.4 ± 0.005 μg/ml, however ascorbic acid exhibited comparatively lower IC$_{50}$ computed to 12.5 ± 0.004 μg/ml as tabulated in Chapter no 5.2, Table no. 5.2.4.8.1.1. The comparison graphs are shown in Chapter no 5.1, Figure no. 5.1.4.8.1.1. The IC$_{50}$ value thus obtained for hydroalcoholic *M. zapota* leaves extract is higher than that reported by Chanda SV. *et al.*, 2012, for the ethyl acetate, acetone and aqueous *M. zapota* leaves extracts.

**4.8.2 HYDROXYL RADICAL SCAVENGING ACTIVITY:**

The Hydroxyl Radical Scavenging activity was thus estimated by studying the competition between deoxyribose and the extract for hydroxyl radicals. They were generated from the Fenton reaction involving Fe$^{3+}$/Ascorbic acid/EDTA/H$_2$O$_2$ system.

The hydroalcoholic *M. zapota* Leaves extract exhibited high hydroxyl radical scavenging activity compared to bark extract however Gallic acid activity was very high
low concentration as tabulated in Chapter no 5.2, Table no. 5.2.4.8.2.1. The comparison graphs are shown in Chapter no 5.1, Figure no. 5.1.4.8.2.1. The \( IC_{50} \) values for hydroalcoholic leaves and bark extract was 210.5 ± 0.005 µg/ml and 752.44 ± 0.003 µg/ml respectively, whereas ascorbic acid exhibited 86.85 ± 0.046 µg/ml. The hydroalcoholic leaves extract thus exhibited better scavenging activity than that reported by Chanda et al., 2012, for the ethyl acetate and aqueous \textit{M. zapota} leaves extracts.

4.8.3 SUPEROXIDE ANION FREE RADICAL SCAVENGING ACTIVITY:
The hydroalcoholic extracts of \textit{M. zapota} leaves and bark studied experimentally exhibited similar superoxide anion free radical scavenging activity; however the standard, gallic acid exhibited strong scavenging ability comparatively as tabulated in Chapter no 5.2, Table no. 5.2.4.8.3.1. The \( IC_{50} \) values for hydroalcoholic leaves and bark extract reported to a value 205.08 ± 0.004 µg/ml and 265.39 ± 0.002 µg/ml respectively, whereas Gallic acid exhibited 154.95 ± 0.008 µg/ml. The comparison graphs are shown in Chapter no 5.1, Figure no. 5.1.4.8.3.1. The hydroalcoholic leaves extract thus exhibited higher superoxide anion free radical scavenging activity to that of the ethyl acetate extract however the value was comparable to the \( IC_{50} \) value for aqueous \textit{M. zapota} leaves as reported by Chanda SV. et al., 2012.

4.9 PROCUREMENT AND CHARACTERIZATION OF LEMONGRASS OIL:
\textit{Cymbopogon citratus} (DC). Synonym \textit{Andropogon citratus} DC family Poaceae commonly known as Citronella grass, Fever grass, Lemon grass, Lemon-scented grass, West Indian Gandhatrina, Gauti chai, Khawi, Sera Hirva cha. Leaves were obtained from the local garden of St. John Institute of Pharmacy and Research (St. John Technical Campus) District Palghar, Maharashtra, India. The plant is perennial grass comprising leaves growing upto a length of up to 1 meter, width of about 1-1.5 cm, scabrous, flat, long-acuminate, smooth rough to touch and scented.

Leaves of the plant were separated, wiped with clean cloth, coarsely grinded and used for the extraction of volatile oil using Clavengers apparatus. Dr. Harshad M. Pandit,
Head of the Department of Botany, Guru Nanak Khalsa College of Arts, Science and Commerce, Matunga, Mumbai authenticated the plant and placed the specimen no. #100314 in the Herbarium of the Department of Botany, G. N. Khalsa College. The authentication certificate for *Cymbopogon citratus* is given in Chapter no 5.1, Figure no. 5.1.4.9.0.

Preliminary studies were performed using the extracted sample. The extraction efficiency of lemongrass oil from 50 gm of coarse leaves powder using Clevenger's apparatus was found to be just 0.3 % v/v. the yield obtained was very low and the process was time consuming. Thus based on the results the further studies were performed using Lemongrass oil sample procured from Dr. K. S. Laddha, YUCCA Enterprises, Antop Hill, Mumbai. It was further subjected to characterization studies as follows.

**4.9.1 DETERMINATION OF WEIGHT/ML:**
Weight per mL: 0.905 gm/ml

**4.9.2 DETERMINATION OF REFRACTIVE INDEX:**
Refractive Index: 1.479

**4.9.3 DETERMINATION OF OPTICAL ROTATION:**
Specific Rotation: Levorotatory (-3°)

Thus characterization of the lemongrass oil on comparison to the reported reference standard values confirmed that the procured lemongrass oil was of the superior quality and hence can be used in the further studies.

**4.10 SCREENING FOR THE *IN VITRO* SUNSCREEN ACTIVITY OF THE EXTRACTS:**
Exposure of human skin to ultraviolet radiations can be etiologic in production of reactive oxygen species thereby oxidizing the tissue lipids and proteins. Cell membranes are made up of polyunsaturated fatty acids (PUFA) and hence susceptible to oxidation.
Upon oxidation the chain reaction begins, resulting in the breakdown of the PUFA to the peroxy radical. This lipid peroxy radical then becomes a lipid hydroperoxide a non-radical, but this molecule easily decomposes to an aldehyde (malondialdehyde). Thus a single initiation can cascade a chain reaction leading to the peroxidation of the membrane unsaturated lipids. This leads to altered permeability of the cell membrane and hence altered metabolic functions of the cell, finally leading to cell death. Further the proteins may also undergo oxidation either general or site-specific. General oxidation can lead to aggregation and fragmentation of the proteins and modification of all amino acid residues. Site specific oxidation of proteins occurs when ROS such as hydroxyl radicals are formed at the putative metal binding sites in proteins/DNA/cell membrane thereby inducing site specific damage and thereby susceptible to chemical fragmentation, inactivation and increased proteolytic degradation (Bandhopadhyay et al., 1999).

To overcome such cell membrane structure damage it is therefore essential to provide antioxidants to the skin. The skin membranes endogenously is able to synthesize some of the antioxidants like glutathione or ubiquinol-10 however some external supply of vitamin C, vitamin E etc. molecules is required in adequate quantities to combat the oxidative stress. Although ROS generated oxidative stress is associated with deleterious processes and harmful effects within a biological system, it is also essential to the existence and development of the cell. Low levels of ROS participate in biochemical processes such as intracellular messaging in cell differentiation, cell proliferation, growth arrest and even apoptosis. Hence loading a cell with a high dose of antioxidants might scavenge too many ROS and essential function such as killing of invading bacteria is inhibited. Thus a multi-scavenger system could be used so as to combat the oxidative damage to the cell in appropriate concentration. Phyto-antioxidants thus play an important role as they have been reported to exhibit multi-scavenging ability. They have also been reported to provide adequate permeability thus protecting the skin from the harmful ultraviolet radiations in the deeper layers. Hence based on the antioxidant activity observed in the above results, the hydroalcoholic extract of *M. zapota* leaves and bark could be presumed to serve as effective photoprotectives.

**4.10.1 EFFECTIVE ABSORPTION SPECTRA MEASUREMENT:**
Ultraviolet spectrum obtained using hydroalcoholic *Manilkara zapota* leaves extract gave the $\lambda_{\text{max}}$ at two peaks as 274 nm and 264 nm wavelength, further the absorbance was fairly good from 290-380 nm which is specifically UV-B and UV-A region. The spectra obtained from the leaves extract covered a wide wavelength with good absorbance at the same concentration than the bark extract. Lemon grass oil also showed a $\lambda_{\text{max}}$ at two peaks as 272 nm and 281 nm extending further with a good UV radiation absorption from 290-380 nm. Further combination of the leaves extract and lemongrass oil samples led to a bathochromic shift in $\lambda_{\text{max}}$ and exhibiting three peaks as 324 nm, 287.5 nm, 275.5 nm respectively and the spectrum showed very good UV absorption in the range 290-380 nm. Para amino benzoic acid and benzophenone used as a standard sunscreen agent showed a $\lambda_{\text{max}}$ at 265 and 257 nm respectively; also the curve degrades very rapidly upto 310 nm thereby showing negligible absorbance over 320 nm. The spectrums of the marketed formulations EKRAN 30® lotion and SUNKARE 50® Gel exhibited a $\lambda_{\text{max}}$ at 309 nm and 308.5 nm wavelength respectively, further the absorbance in both spectrums was very good upto 350 nm. Hence it was decided that the leaves extract could possess a potential sunscreen activity like the marketed formulations. The effective absorption spectra of the extracts, the standards and the marketed formulations are shown in Chapter no 5.1, Figure no. 5.1.4.10.1.1. Thus the further photostability was determined using leaves extract in combination with lemongrass oil. The results thus obtained proved to be beneficial in determining the potential sunscreen agents obtained from herbal source.

### 4.11 DETERMINATION OF PHOTOSTABILITY OF THE EXTRAITS USING *IN VITRO* PHOTOTOXICITY TESTS:

#### 4.11.1 CANDIDA YEAST TEST:

The yeast test using *Candida albicans* as developed by Daniels is the most simple *in vitro* phototoxicity test. This test is based on a simple principle wherein the photodegraded products/metabolites of the phototoxic drugs formed as a result of UV exposure affects the growth of *C. albicans*. However the photostabilized formulation does not yield any metabolites as such and hence the growth of *C. albicans* remains unaffected.
Candida yeast test revealed that *M. zapota* leaves extract on irradiation did not produced any phototoxic compounds hence the growth of *C. albicans* was not affected. However the known phototoxic drug psoralen containing Melanocyl® cream yielded phototoxic compounds on irradiation which affected the growth as shown in Chapter no 5.1, Figure no. 5.1.4.11.1.1. The dark control samples remained unaffected and a tremendous *C. albicans* growth was observed in case of Melanocyl® cream. The results thus indicate the photostability of the *M. zapota* leaves extract. *Candida albicans* seeded agar plate used as a negative control also showed an unaffected growth on exposure thus assuring that the growth inhibition was not due to toxic UV radiations but merely due to formation of toxic metabolites of UV irradiated psoralen containing formulation.

### 4.11.2 PHOTOSTABILITY STUDY USING SPECTRA MEASUREMENT:

UV Spectra measurement revealed that on irradiation of the test sample i.e. *Manilkara zapota* leaves extract did not show a significant change in the absorption spectra particularly in the wavelength range 260-350 nm however the para amino benzoic acid (PABA) sample was affected to a major extent in the wavelength range 260-300 nm as shown in the Chapter no 5.1, Figure no. 5.1.4.11.2.1, indicating its photo instability. However the benzophenone spectra remained unaffected indicating high photostability. Marketed formulations, SUNKARE 50®Gel, EKRAN 30® Lotion also revealed no significant change in the spectra. Lemongrass oil alone was found unstable on irradiation however combination of leaves extract and lemongrass oil led to its photostabilization.

### 4.11.3 PHOTOSENSITIZED PEROXIDATION OF LINOLEIC ACID:

UV-A radiation constitutes a very significant oxidative stress by causing peroxidation of lipids in biological systems (Bose, B., and Chatterjee, S. 1995). Membrane phospholipids more specifically the polyunsaturated fatty acids are prone to a degenerative lipid peroxidation process. A secondary product of lipid peroxidation which are toxic aldehydes such as malondialdehyde (MDA), formation is a general mechanism of this process. Further the secondary product produces widespread changes in cellular membranes by reacting with protein and non-protein substances. A widely used sensitive
method to determine the extent of lipid peroxidation in animal tissues is thus based on the reaction of lipid peroxides with thiobarbituric acid (TBA). Linoleic acid is one such fatty acid and linoleic acid hydroperoxide obtained by peroxidation reacts with TBA to yield the same red pigment as that obtained with MDA (Onoue et al., 2006).

Photo labile drugs are known to cause lipid peroxidation in membrane lipids due to the degraded metabolites, leading to formation of ROS in lipids. ROS is also one of the major causative factors for increased MDA formation. Thus measurement of extent of such hydroperoxide formation with plain extract on exposure to UV radiation will provide a great input in determining the degree of photostability of the extracts. Linoleic acid lipid peroxidation test revealed that the extract on irradiation produced less MDA, a peroxidation product comparable to standards quercetin and gallic acid which are the effective flavonoids and polyphenols used as photo protective and antioxidant, indicative of its photostability. A standard curve of 1,1,3,3-tetrethoxypropane was used to determine the extent of peroxides formed as shown in Chapter no 5.1, Figure no. 5.1.4.11.3.1. The readings closely resemble the marketed product FACEGUARD® containing photostable synthetic sunscreens, Uvinul A Plus (Diethylamino Hydroxybenzoyl Hexyl Benzoate), Tinosorb S (Bemotrinizol) and Octinoxate as shown in Chapter no 5.2, Table no. 5.2.4.11.3.1.

The results thus obtained from the in vitro phototoxicity studies conducted revealed the potential photostable sunscreen activity of the hydroalcoholic M. zapota leaves extract. The phytoconstituents responsible for such activity were mainly the flavonoids, coumarins and polyphenolic compounds which are able to absorb the ultraviolet radiations as they have been confirmed in the earlier results. Reports also reveal that above compounds are also able to scavenge the free radicals, specifically flavonoid like compound exhibiting anti-inflammatory, cardioprotective and anticancer by virtue of its free radical scavenging activity (Middleton et al., 2000). Flavonoids have been reported in the literature to possess wide pharmacological actions, the major activity pertaining to the present research is the prevention against UV radiations induced skin damage. Topical applications of quercetin, hesperitin, and naringenin have provided protection against photooxidative stress. Thus the phytoconstituents like flavonoids, coumarins and polyphenols obtained from the hydroalcoholic M. zapota leaves extract
can be considered to be the main constituents responsible for the antioxidant activity and photostable sunscreen activity. Addition of lemongrass oil further enhanced the sunscreen activity of the hydroalcoholic *M. zapota* leaves as observed from the results; this volatile oil mainly contains monoterpenes like citral. Hence it can be further stated that the monoterpenes can potentiate the sunscreen activity of the flavonoids, coumarins and polyphenol like constituents.

The herbal sunscreen formulations available in market today are found to contain a combination of extracts which constitute multi-phytoconstituents. Extracts obtained from *Luffa cylindrica*, *Portulaca oleracea*, Cucumber, White lily flower (*Crinum asiaticum*) have been reported to possess sunscreen activity. Also Raspberry seed oil, Avocado oil, Macademia nut oil, Almond oil, Shea butter oil and Jojoba oil possess sunscreen activity. Moreover the herbal extracts are also combined with the synthetic sunscreen agents to broaden the ultraviolet absorption spectra thereby providing greater protection. A detail note on the marketed formulations comprising combination of multi extracts and/or synthetic components is given in Chapter no 5.2, Table no. 5.2.4.11.3.2. The observed results are thus higher than the single extract or single phytoconstituent; thereby gaining greater consumer acceptance. The higher results could be attributed due to the synergistic activity of these multi constituents. Hence in the present research work the hydroalcoholic extract obtained from *M. zapota* leaves was not subjected for isolation of the individual constituent and formulate the product comprising single phytoconstituent. It will not only lessen the overall ultraviolet absorptive range but also may be required in more concentration which could further add to formulation problems. Also due consideration was given to the greater effectiveness of the extract hence addition of lemongrass oil was put forth and the results obtained satisfied the purpose.

### 4.12 SCREENING FOR ANTI-ACNE ACTIVITY:

#### 4.12.1 CUP-PLATE METHOD:

Cup plate method using *S. epidermidis* and *P. acnes* revealed that the hydroalcoholic extract of the *M. zapota* leaves and bark exhibited a growth inhibition against the organisms. Thus the antiacne activity can be established as per the prediction.
The experimental set up for the aerobic incubation condition of the *S. epidermidis* seeded petriplates is shown in chapter no 5.1, Figure no. 5.1.4.12.1.1. The Figure no. 5.1.4.12.1.2 in Chapter 5.1, shows the zones of inhibitions observed in antiacne studies against *S. epidermidis* while those against *P. acnes* are shown Chapter 5.1, Figure no. 5.1.4.12.1.3. Further the experimental set up for maintaining the anaerobic conditions is shown in Chapter 5.1, Figure no. 5.1.4.12.1.4. The zone of inhibitions thus measured using the leaves extract against *S. epidermidis* and *P. acnes* was found to be greater than that of the bark extract at the increasing concentrations as shown in comparison bar graphs of Chapter no 5.1, Figure no. 5.1.4.12.1.5 and 5.1.4.12.1.6 respectively. The highest zone of inhibition was achieved at 1% w/v and 2% w/v respectively. Although the zone of inhibition at 4% w/v were found to be slight higher and nearly comparable to that of 2% w/v but such high concentration of the extract possessed a solubility problem. Thus the 4% w/v concentration was not used further. The zone of inhibition obtained using Clindamycin and Erythromycin were comparatively very high at the same concentrations. Further addition of 0.5% v/v lemongrass oil in appropriate ratio to the individual hydroalcoholic *M. zapota* leaves and bark extracts separately increased the diameter of the growth inhibition compared to just lemongrass oil as shown in Chapter no 5.2, Table no. 5.2.4.12.1.1 and 5.2.4.12.1.2 respectively. The various proportions in which the lemongrass oil added were; extract: Lemongrass oil in the ratio of 1:1, 1:10, 75:25, 25:75 etc. However the ratio of extract: Lemongrass as 1:1 gave the most promising zone of inhibitions. Hence the ratio 1:1 was used in the further research work. The results thus obtained on combining the above key components could be attributed to the synergistic activity of the hydroalcoholic leaves and bark extract individually along with lemongrass oil.

The leaves extract thus exhibited higher growth inhibitions against both organisms and on addition of lemongrass oil the zone of inhibitions was greater than that of zone of inhibitions showed by the standard antibiotic Erythromycin. Also the results obtained using marketed formulation FACECLIN® Cream was comparable to that of the leaves extract. Thus it was decided worthwhile to proceed further towards formulation development using only the leaves extract and lemongrass oil rather than the bark extract. The presence of flavonoids, coumarins, polyphenols, terpenoids and saponin glycosides
in the hydroalcoholic leaves and bark extract of *M. zapota* could be attributed for such activity. The ethyl acetate extracts leaves and bark extract of *M. zapota* have been reported to exhibit antimicrobial activity in earlier literature due to the presence of similar constituents (Karim *et al.*, 2011) hence the above result extends their effectiveness spectrum in the antiacne area as well. Methanolic extract from *Camellia sinensis* was confirmed to exhibit antiacne activity against *S. aureus*, *S. epidermidis* and *P. acnes* (MIC: 1.25 mg/ml) by comparison to that of the extracts of *Glycerrhiza glabra* and *Calendula officinalis* due to presence of alkaloids, flavonoids, glycosides and terpenoids. The flavonoids like Kaempferol obtained from cassia flowers have been reported to exhibit effective antiacne activity against *P. acnes*. Thus the above results could also be attributed to the presence of flavonoids, alkaloids, terpenoids and glycosides (Sinha *et al.*, 2014). Lemongrass oil comprising mainly the monoterpenes like citral, geranial, limonene are already reported earlier as antiacne agent; hence the judicious decision to include the oil has led to potentiate the therapeutic activity. Herbal extracts when used as whole rather than the isolated phytoconstituent has been reported to exhibit synergistic activity by the multi-phytoconstituents thereby enhancing the therapeutic activity. The whole extracts obtained from *Echinacea purpurea*, *Garcinia mangostana*, *Eupatorium odoratum*, *Camellia sinensis* etc. have been reported to possess strong inhibitory activity against *P. acnes*, *S. epidermidis*, *S. aureus* etc. In addition to these *Senna alata* (MIC: 0.625-2.5 mg/ml) and *Barleria lupulina* (MIC: 1.25-2.5 mg/ml) also showed strong inhibitory activity against acne causing organisms. The marketed herbal antiacne formulations majorly comprise poly herbal extracts or poly-phytoconstituents thus producing greater effectiveness. Hence in the present research work the hydroalcoholic extract obtained from the *M. zapota* leaves was not subjected for isolation of the single phytoconstituent but used as a whole for the study.

### 4.12.2 AGAR DILUTION METHOD:

The agar dilution method using hydroalcoholic leaves and bark extract of *M. zapota* individually revealed a respective MIC value of 0.05 % w/v and 0.2 % w/v against *S. epidermidis* also against *P. acnes* the MIC value was 0.1 % w/v and 0.2 % w/v which was comparatively very high to that of the standard antibiotics currently used to treat acne
conditions. The photographs showing the minimum inhibitory concentrations determination are shown in Chapter no 5.1, Figure no. 5.1.4.12.2.1. Although he concentrations are higher than those of the standard antibiotic the results obtained could be beneficial to supplement the current antiacne remedies and reduce the overall irrational use of antibiotics.

The anti acne studies conducted in the present research thus confirmed that the plant extracts could serve as effective and potential antiacne drugs. This will help combat the growing incidences of bacterial resistance, side effects and provide greater consumer satisfaction.

4.13 PHARMACEUTICAL FORMULATION DEVELOPMENT:
The hydroalcoholic extract obtained from the *M. zapota* leaves thus was found to possess sunscreen activity as well as antiacne activity against *S. epidermidis* and *P. acnes*. However the extract cannot be applied as such on the skin surface hence a suitable topical formulation was desired. Formulating a cream was thought more appropriate due to greater elegance of the product and hence consumer preference. Further formulating a W/O cream could led to more occlusive action on application and promote more sweating especially in the summer season and hot humid conditions, may make the face oily on application thus O/W cream could be more acceptable. Considering all these aspects a stable O/W cream was formulated. Oil in Water (O/W) cream thus formulated using hydroalcoholic extract of *M. zapota* leaves by standard fusion method was elegant to view. Triethanolamine stearate used as an *in situ* emulsifier produced a smooth product. Five different products were formulated each varying in composition of the excipients. The details of each formula are given in Chapter no 5.2, Table no. 5.1.4.13.1.

4.14 CHARACTERIZATION OF THE FORMULATION:

4.14.1 APPEARANCE AND TEXTURE:
Appearance of the 1% w/w cream formulations revealed very good light greenish colored more specifically “Arylide yellow” colored smooth textured formulations exhibiting very good luster as shown in Chapter no 5.1, Figure no. 5.1.4.1.1

4.14.2 SKIN STAINING:
The 1% w/w cream comprising hydroalcoholic extract of M. zapota leaves did not produce any stain on the skin as shown in the Chapter no 5.1, Figure no. 5.1.4.2.1. The extract contained flavonoids and hence expected to stain the skin surface as flavonoids are colored compounds, however the photographs revealed that at the concentration of the extract which was used in the cream there was no staining observed. However the same extract used in the crude form did stain the skin slightly when compared to the color of the skin prior application.

4.14.3 ODOR:
The human volunteers used in the study provided a useful and similar description of the product’s odor. The odor of all the cream formulations as described by the volunteers was that of a typical lemongrass perfumed formulation.

4.14.4 pH:
The creams exhibited a pH around 6.8, the details of the individual product is tabulated in Chapter no 5.2, Table no. 5.2.4.1.4.1.

4.14.5 HOMOGENEITY:
Visual observation and microscopic examinations revealed that the formulation F4 was homogenously mixed, absence of agglomeration and complete dispersion of other excipients as shown in the Chapter no 5.1, Figure no. 5.1.4.5.1. However F1 and F5 showed some lumps of the active ingredient and wax system. This could be attributed to the concentrations of excipients added. Hence formula F1, F3 and F4 were continued for further studies.

4.14.6 SPREADABILITY:
For a good spreadability, the cream should behave like a good lubricant exhibiting maximum slip and minimum drag. Lesser the time taken by two plates to cover a fixed distance, lesser the friction between the glass surfaces and the cream, more spreadable is the cream. The glass plate containing 0.5 gm of cream was able to slide in less than 10 seconds a distance of 5 cm, thus indicating a very good spreadability index.

4.14.7 **IN VITRO DIFFUSION STUDIES:**

In vitro Diffusion studies of the F4 cream comprising 1%w/w of hydroalcoholic extract of *Manilkara zapota* leaves and 0.5 % v/v Lemongrass oil (1:1) was evaluated in triplicate. Three Franz diffusion cells were set at a temperature of 37 ± 0.5º C and a formulation weighing 0.5 gms was placed on the skin tissue. The experimental set up is shown in Chapter no 5.1, Figure no. 5.1.4.14.7.1.

The cream formulation on analyses gave three peaks at 310 nm, 279.5 nm and 255.5 nm. The change in the peaks as that of the *M. zapota* leaves extract and lemongrass oil combination (324 nm, 287.5 nm and 275.5 nm) could be due to the change in the solvent, as in the study procedure phosphate buffer pH 7.0 is used. Thus the concentration of the constituents diffused across the skin was measured at three different wavelengths. Hence the standard curves used to determine the constituent concentration and hence the percent permeated were also three. The equations used were \( Y=0.035X + 0.02 \) at 310 nm, \( Y= 0.024X+0.05 \) at 279.5 nm and \( Y=0.0058X+0.0036 \) at 255.5 nm. The observations of the test are tabulated in Chapter no 5.2, Table no. 5.2.4.14.7.1. The graph showing in vitro permeation extent of the actives in the cream is shown in Chapter no 5.1, Figure no. 5.1.4.14.7.2. The constituents absorbing at 310 nm could diffuse upto 58%, constituents absorbing at 279.5 nm could diffuse upto 38% and that of constituents absorbing at 255.5 nm could diffuse upto 57%. The diffusion studies revealed that the formulation containing the extract and lemongrass oil could diffuse to a maximum value of 50-60% the constituents at the end of 10 hours indicating it"s retention at the superficial layer and thereby ability to provide maximum protection to the superficial skin.

4.14.8 **RHEOLOGY:**
Rheological characteristics of the cream determined using Brookefield Viscometer RV model showed an increase in viscosity at less than a linear rate with increasing shear rate. Also, increasing the shear rate caused deformation of the cream which was not regained completely during the down curve or as the shear was decreased. Thus the Up curve and Down curves were not superimposable. Hence the formation of Hysteresis loop and thus the cream is interpreted to exhibit a non-Newtonian pseudoplastic rheology. The observations of the rheological data are tabulated in Chapter no 5.2, Table no. 5.2.4.14.8.1. The graph showing rheology of the cream is given in Chapter no 5.1, Figure no. 5.1.4.14.8.1.

4.14.9 STABILITY STUDIES:
Three batches of the cream formulations were kept at 40°C and 25°C for a period of six months as required by ICH Q1A R2 guidelines and also refrigeration condition. The formulations were evaluated at regular time points for visual changes like color, appearance, also odor, visual appearance (separation of phases), drug content and pH. The observations of the stability study are tabulated in Chapter no 5.2, Table no. 5.2.4.14.9. More specifically each temperature conditions are recorded in Chapter no 5.2, Table no. 5.2.4.14.9.1, 5.2.4.14.9.2 and 5..2.4.14.9.3 respectively. All the cream formulations were found to be stable at room temperature (25°C) as well as at 40°C during 6 months evaluation duration and revealed no signs of instability like, separation and drastic content difference, however there were slight changes in the drug content on six month evaluation. The drug content analyses were performed at three different wavelengths as 324 nm, 287.5 nm and 275.5 nm. The constituents retained in the formulation at the end of six months were determined using the standard curves as Y=0.0583X+0.0036 at 324 nm, Y=0.0041X-0.051 at 287.5 nm and Y=0.0081X+0.05 at 275.5 nm. The spectrums observed at periodic intervals are shown in Chapter no 5.1, Figure no. 5.1.4.14.9.1. These results could be attributed to the loss of lemongrass oil from the formulation due to evaporation. Although the change in drug content was not significant as not more than 10 decrease in the contents was observed, but the evaporation of lemongrass oil should be addressed in the future research work as it may
narrow down the overall ultraviolet absorption spectra and anti acne activity which was potentiated due to its addition.

Based on the above elaborate studies conducted formula F4 revealed the acceptable characteristics hence it was selected for further studies. Further the F4 cream was evaluated for microbial load at the end of six months and found to exhibit microbial stability since as per the limit stated in Indian Pharmacopoeia, 2007 not more than 1000 cfu/gm (colony forming units per gram of cream) of the microbial load was detected in the cream.

4.15 DETERMINATION OF IN VITRO SUN PROTECTION FACTOR (SPF) OF THE FORMULATION:

The evaluation of sunscreen activity of the formula ion would be incomplete without determination of its SPF value. It measures the length of time a sunscreen formulation protects the skin against sunburn, pigmentation and irritation compared to the time taken to redden the skin without its application. Such reactions produced on uncontrolled exposure over a period of time can lead to premature ageing of the skin thereby producing wrinkles. Further the deleterious effects like oxidative stress beneath the epidermal layers could also proceed towards skin cancers. Hence the skin protection is must in the hot climatic habitat. Indian climate more or less in every part of the nation is sunny for atleast 7-8 months hence application of sunscreen is regular for people. An individual"s response to UV exposure varies dependent on his/her skin type. Also many products are available in the market but exactly which one to choose is a challenge for every consumer. The SPF value thus helps to provide a guide in choosing one of them. Thus the present formulation was evaluated for its SPF value and appropriate rating.

The SPF Analysis was performed using UV-2000S Ultraviolet Transmittance Analyzer at Quality Assurance Unit of The Kelkar Education Trust"s, Scientific Research Centre, Cosmeceutical Division, Mulund, Mumbai, by Ms. Rutuja Patil, HOD, Quality Assurance and Ms. Yamini Patil, Junior Scientist.
Principle:
The principle is based on the sample transmittance measurement, where transmittance is defined as the ratio of the illumination passed through a sample to the illumination impinging on the sample.

The cream formulation thus prepared gave a fairly good SPF value of 2.70, however the UV-A/UV-B ratio was found to be 0.662 which means a good protection factor in UV-A as well as in the UV-B region. Also the critical wavelength determined was 385.11. The boots star rating of the cream was found to be 3 (***). The boots star rating is based on the average of UV-A/UV-B ratio. A different rating is given to a range of values as shown below.

<table>
<thead>
<tr>
<th>Mean UV-A/UV-B ratio</th>
<th>Star Rating Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 to 0.59</td>
<td>No Rating</td>
</tr>
<tr>
<td>0.6 to 0.79</td>
<td>***</td>
</tr>
<tr>
<td>0.8 to 0.89</td>
<td>****</td>
</tr>
<tr>
<td>0.90 and over</td>
<td>*****</td>
</tr>
</tbody>
</table>

The details of the observations are given in Chapter no 5.2, Table no. 5.2.4.15.1. The triplicate scans observed on the Analyzer are shown in Chapter no 5.1, Figure no 5.1.4.15.1.1, 5.1.4.15.1.2 and 5.1.4.15.1.3 respectively. The SPF value for sunscreen above 2 is considered as having good sunscreen activity. Kale et al. reported the SPF value of *Murraya koenigii* essential oil as 2.04, *Caltropis gigantean* seed oil as 1.14, *Ocimum sanctum* leaf oil as 1.19 to be good. In the present study the SPF value 2.70 and Boots star rating 3 indicates that the formulation possess good sunscreen i.e sun protecting ability. Thus it could be used as a potential sunscreen agent or also can serve as an adjuvant to be combined with other extracts to exhibit further enhancement of the activity.
4.16 VALIDATION OF THE FORMULATION AS PER BUREAU OF INDIAN STANDARD (BIS) SPECIFICATIONS:

The formulation so prepared has to be validated for standard quality as the guidelines laid down and standards prescribed by the regulatory bodies. The prepared cream formulation being comprised of a plant extract which is effective against acne condition could as per the description and ability to inhibit the growth of microorganism also called as pharmaceutical dosage form however since the application is topical thus referred as cosmeceutical formulation. The standards for such products are laid down by the Bureau of Indian standards (BIS). Hence the formulation was validated to possess the required quality using BIS standards, specifications for skin creams IS 6608:2004.

**Specifications for skin creams:**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal stability</td>
<td>No oil separation or phase separation.</td>
</tr>
<tr>
<td>pH</td>
<td>4-9</td>
</tr>
<tr>
<td>Total fatty substance</td>
<td>5 minimum. (Percent by mass)</td>
</tr>
<tr>
<td>Total residue</td>
<td>10 minimum. (Percent by mass)</td>
</tr>
<tr>
<td>Heavy metals as Pb, max</td>
<td>20 (Parts per million)</td>
</tr>
<tr>
<td>Arsenic, max</td>
<td>2 (Parts per million)</td>
</tr>
<tr>
<td>Microbial content/limit</td>
<td>Total viable count, not more than 1000cfu/g</td>
</tr>
</tbody>
</table>

4.16.1 THERMAL STABILITY:

The product revealed a good thermal stability and hence passes the test when exposed to 45±1°C for 48 hours. The product did not show any oil separation or phase separation.

4.16.2 DETERMINATION OF pH:

The pH of the formulation was found to be 6.8 which within the limits 4-9 hence passes the test.
4.16.3 DETERMINATION OF TOTAL FATTY SUBSTANCE CONTENT:
The total fatty substance was 15 mg/100 g cream which is above the minimum limit 5, hence passes the test. This test basically helped to understand the extent of fatty acid present. More the extent, more oily is the product; hence the feel of the product on application to skin may affect the product acceptability. Since the fatty substance value was found to be low, the formulated cream does not exert greasy feel.

4.16.4 DETERMINATION OF RESIDUE:
The total residue was estimated which exceeds the minimum value 10 hence the product passes the test.

4.16.5 LIMIT TEST:
4.16.5.1 Microbial Limit Test of the extract and the cream:
The product passes the microbial limit since as per the limit not more than 1000 cfu/gm of the microbial load detected.

4.16.5.2 Lead:
The product passes the limit test since the opalescence produced by test solution was very much less than the standard lead solution of 20ppm.

4.16.5.3 Arsenic:
The product passes the test since the stain produced by the test sample was not intense than that of the standard stain of 2 ppm.

Thus the cream was found to be of good quality as it passes the test specifications.

4.17 CUTANEOUS TOXICITY EVALUATION:
4.17.1 ACUTE DERMAL IRRITATION TEST:
The formulation was applied (0.4g) as a non-occlusive dressing on dorsal shaved area (approx. 1 cm²). The experimental set up to hold the animals after applications of the formulations and observations of the skin reactions are shown in Chapter no. 5.1, Figure no. 5.1.4.17.1.1. There were no unscheduled deaths during the study period. No test formulation related clinical signs and significant body weight changes were observed during the study period. The summary of body weights are tabulated in Chapter no. 5.2, Table no. 5.2.4.17.1.1. The skin reactions were scored using Draize’s score and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.1.2. The scores of skin reaction as erythema and edema were observed as “0” in four animals however one of the animal showed an exceptional higher value of 4. It could be due to weak immune system of the animal exhibiting greater irritation hence higher erythema signs.

The histopathological examination of skin samples were performed by Dr. Sanjay Pawar, M.V.Sc. Pathology Registration no. 7238. The histopathology of formulation site did not reveal any lesions of pathological significance and was found to retain normal physiology as that of the control site. The test formulation containing 1% w/w M. zapota leaves extract cream thus was concluded to show no major toxicological dermal reactions like erythema and edema as seen from histopathological examination of skin at the end of 14 days as shown in Chapter no. 5.1, Figure no. 5.1.4.17.1.2. This also proves that 0.5% w/v concentration of lemongrass oil was also safe to use and non-irritant.

4.17.2 ACUTE DERMAL TOXICITY:
Topically applied formulations need to be tested for acute dermal toxicity and hence the present study was planned. The formulation (0.3 g) of each dose (Dose 1, 1000mg/kg and Dose 2, 2000mg/Kg of the extract) was applied as a non-occlusive dressing on dorsal shaved area (approx. 1 cm²). Observations of the skin reactions in animals are shown in Chapter no. 5.1, Figure no. 5.1.4.17.2.1. There were no unscheduled deaths in any groups during the study period. No test formulation related clinical signs and significant body weight changes were observed in any group I i.e Test dose 1 and group II i.e Test dose 2 animals during the study period as reported in Chapter no. 5.2, Table no. 5.2.4.17.2.1 and 5.2.4.17.2.2 respectively. The grading of the skin reaction as observed during the test is
summarized in Chapter no. 5.2, Table no. 5.2.4.17.2.3 for Test dose 1 animals. It was noted that as per the Draize’s scoring system, one of the two animals out of five scores a rating of 2 for erythema and edema reactions however one animal scored a rating of 4. This indicated a mild reaction in two animals and a moderate reaction in one animal. The organ weights of the animals were obtained after sacrificing the animals at the end of 14 day study and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.2.4. The organ to body weights were also determined and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.2.5.

The grading of the skin reaction for Test dose 2 animals as observed during the test is summarized in Chapter no. 5.2, Table no. 5.2.4.17.2.6. It was noted that as per the Draize’s scoring system, only one of the five animals scored a rating of 3 for erythema and edema reactions thereby indicating a moderate reaction in one animal. The organ weights of the animals were obtained after sacrificing the animals at the end of 14 day study and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.2.7. The organ to body weights of group II animals were also determined and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.2.8. Further a comparison of organ to body weights were made in both the test doses together and tabulated in Chapter no. 5.2, Table no. 5.2.4.17.2.9. On statistical analysis using student’s t-test at a 95% confidence limit the ratios was found to be non-significant with respect to each other.

The histopathological examination of skin samples of formulation site did not reveal any lesions of pathological significance and was found to retain normal physiology as that of the control site. The observations of the histopathology study for test dose 1 and test dose 2 are reported in Chapter no. 5.1, Figure no. 5.2.4.17.2.2 and Figure no. 5.2.4.17.2.3 respectively. There were no abnormal findings on gross necropsy in both groups on day 15 after the treatment. However some infiltration was seen, hence it was concluded that the test formulation at dose 1 shows mild dermal reactions while test formulation at dose 2 shows moderate dermal reactions as inferred from this test. The irritation reactions could be due to the presence of constituents like tannin which are known to be skin irritants. Lemongrass oil comprises citral which is reported to be a primary skin irritant (Edris A. 2007) at a concentration above 5 % v/v hence the concentration of the oil in the cream formulation was very low, just 0.5 % v/v. The test
doses applied on animals as per the guidelines prescribed were much higher than the actual concentration which is included in the cream product i.e. 1% w/w. So such higher dose could definitely contain higher tannins which could be giving slight skin irritation as per the observation. Statistical analysis of the organ to body weight obtained after sacrificing the animals and excising the major organs from the formulation applied animals also did not reveal a significant change. Thus it can be concluded that the dermal application of test formulation did not show any mortality or gross pathological effects in rats in acute dermal toxicity study conducted as per OECD guideline number 434, hence can be considered as safe to use in humans.

FORMULATION OF A NUTRACEUTICAL PRODUCT CONTAINING MANILKARA ZAPOTA (L.) PLANT FRUITS:

Hippocrates many years back had given a health key stating, “Let thy food be thy medicines and thy medicines be thy food”. The key holds so truth that for the centuries earlier and further years to come its very much applicable. Indian, Egyptian, Sumerian, and Chinese civilization has provided the evidences about the health key put forth by Hippocrates. Ayurveda, the 5000 years old, ancient health science have reported the benefits of fruits for therapeutic purposes. Chyavanprasha, Brahma Rasayana, Phala Ghrita, Arjuna Ksheerapaka, Shatavari Ghrita, Rasona Ksheerapaka are some of the Ayurvedic nutraceuticals. The broad classes of the nutraceutical components include the antioxidants, dietary fibres, polyunsaturated fatty acids, probiotics and prebiotics. These components have been reported to provide cardioprotective, cancer protective, hepatoprotective, wound healing etc. beneficial effects for years. Modern nutraceutical industries realized the tremendous potential of fruits to heal the ailments, the deficiencies of consumers eating habits and thus began to bloom as the processed fruits were made available ready to the consumers. Later the nutraceutical foods were synonymously called as designer foods, health foods, fortified foods, pharma foods, functional foods and dietary supplements. Indian market is growing at the rate of 21% per year. AMWAY has the highest share of about 15% and the other major industries include Dabur, Ranbaxy
and Pfizer. Thus these nutrifirms are bridging the gap between food and the medicine. The research in these areas are thereby increasing.

*M. zapota* fruits commonly called as chickoo fruit is a tropical fruit with is a grown in India and is very sweet and nutritive. It contains very good amounts of antioxidants, vitamins and elements. Phenolic compounds are the major antioxidants present in fruits which are responsible for free radical scavenging activity. Ascorbic acid is also another free radical scavenger present in fruits. Dietary fibres in food also are an essential requirement in foods. Insufficient consumption of dietary fibres can produce physiological problems like constipation, increased risk of coronary heart disease, fluctuation of glucose and insulin levels etc. (Mahattanatawee et al., 2006). Dietary fibre intake can be increased in the diet by increasing the intake of fruits and vegetables adequately in daily diet. They are also reported to prevent cancers. The National Research Council thus set the “Dietary Reference Intake” (DRI) for the first time determining the dietary fibre intake. The “adequate intakes” (DI) for dietary fibres are given as 14 g per 1000 calories. Chikoo fruits contain all the above nutritive components hence can serve as a nutritional fruit. The fruits are also a good source of energy as it contains carbohydrates and sugars. A detailed description of the components of the *M. zapota* fruits is given in Chapter no. 5.2, Table no. 5.2.4.19.0. However it ripens and decays very fast, within 7-8 days at ambient temperature. Hence the nutritive value is lost. Thus attempts were made to delay the ripening process by application of inhibitor 1-methyl cyclopropene (1-MCP) (Jiang et al., 2006). It has been noted that the consumers are nowadays aware of such artificial treatments of the fruits and hence raised the safety issues. Moreover the consumer acceptance of such fruits which are stored for long time in an unnatural method is unhealthy. Hence in the present research work attempt is made to obtain a stable product and safe for ingestion.

### 4.18 PROCUREMENT OF MANILKARA ZAPOTA (L.) PLANT FRUITS:

*M. zapota* fruits were obtained from St. John Technical Campus, Palghar, Maharashtra, India and authenticated by Dr. Harshad M. Pandit, Head of the Department of Botany, Guru Nanak Khalsa College of Arts, Science and Commerce, Matunga, Mumbai. The
specimen no. #nlr 080314 is deposited in the Herbarium of the Department of Botany, Guru Nanak Khalsa College, Matunga, Mumbai, Maharashtra, India. The authentication certificate for *M. zapota* plant is given in Chapter no. 5.1, Figure no. 5.1.4.2.1 as mentioned earlier. The *M. zapota* plants are grown specifically in the Campus for its fruit. Thus the availability of the fruits for the research work was plenty and also cheap.

4.19 PREPARATION AND STANDARDIZATION OF *M. ZAPOTA* FRUIT JUICE:

The prepared pulp of the ripe fruits was used for the initial assays and standardization procedure as follows.

4.19.1 pH:

The ripe fruit pulp was found to exhibit pH 3.9 on dilution with water during its measurement. This indicates that the fruit pulp was acidic hence could comprise the acidic components like tannins to a greater extent.

4.19.2 TOTAL SOLID CONTENT:

Solid content of the ripe fruit pulp was determined to be 15% w/w. Considering the overall fruit size, and comparatively less solid content it could be noted that the fruits comprises more of water content.

4.19.3 ASCORBIC ACID CONTENT:

Ascorbic acid content of fresh pulp was found to be 0.176% w/w on titration with 0.05 M Iodine solution using starch as an indicator. Ascorbic acid is a very good free radical scavenger hence *M. zapota* fruits can be concluded to possess antioxidant activity.

Thus from the preliminary studies conducted on the fresh fruit pulp obtained from *M. zapota* and the results obtained it was decided to proceed with the stable product development. As the earlier reports have already evaluated *M. zapota* fruit pulp for antioxidant activity, presence of polyphenols, carotenoids, elements like Iron, copper, calcium potassium etc. and claimed that the fruit pulp is nutritious (Aradhya *et al.*, 2007).
Also Mahattanatawee et al. have reported the total dietary fibre content, antioxidant ascorbic acid content, total soluble phenol content, pectin content, galactouronic acid content etc. for the sapodilla fruit. Hence in the present study emphasis was given to the stable product development.

4.20 FORMULATION AND CHARACTERIZATION OF THE NUTRACEUTICAL PRODUCT:

Drying is the most commonly used unit operation in Pharmaceutical manufacturing having varying applications as aid in preparing of granules, processing of materials, and preparation of powdered extracts. The unit operation can also be used to reduce the bulk and weight of material which would reduce the overall cost of transportation and storage. Other uses include as the aid in preservation of animal and vegetable drugs, where it minimizes the moisture laden material from mold and bacterial growth. Thus drying phenomena offers more stability to the animal and vegetable drugs (Khar et al., 2013). Thus in the present research in order to obtain a stable product using M. zapota fruit pulp, drying was considered as a suitable option. This would help to prepare a solid product and would provide greater stability than the liquid moist pulp.

4.20.1 SPRAY DRYING:

Spray drying is referred as a method to produce a dry powder from a liquid or slurry by rapidly drying with the aid of hot gas. This is a best suited method to dry a thermally sensitive material such as food and pharmaceuticals. Food products vary in their composition and also in the end product properties and requirements. But the end product thus produced needs to be suitable and safe for consumption in human, hence a spray drier that fulfills the requirements for hygienic design is the most desirable.

Based on the practical requirements the M. zapota fruit pulp was subjected to spray dry. However the spray drying technique did not yield a free flowing solid powder form from the fruit pulp. The pulp contained some sticky mucilaginous mass. One of the most important parameters during drying operation is the total moisture content in the exhaust air. The composition of the product is affecting greatly, eg. A product with high
protein content can be dried at high total moisture content in the exhaust air, while slurry with high carbohydrate content requires low moisture content to avoid sticking of the powder in the chamber, ducts and cyclones. Comparing the protein content and carbohydrate content from the *M. zapota* fruit as reported in Chapter no. 5.2, Table no. 5.2.4.19.0 it can be observed that the carbohydrate content is comparatively very high than that of the protein content. Thus it can be concluded that the high carbohydrate and mucilaginous mass reduces the drying potential of slurry. Hence another drying technique was employed.

4.20.2 FREEZE DRYING:
Many pharmaceutical products lose their viability in the liquid state and could deteriorate readily if dried in air at normal atmospheric pressure. These materials may be heat sensitive or may readily react with oxygen, hence to render them stable they need to be dehydrated to a solid state. The material is first frozen and then subjected under a high vacuum to heat so that the frozen liquid sublimes leaving only the solid, dried component of the original liquid. Such method is called as Freeze drying, lyophilization, Gelsication or drying by sublimation. Many food stuffs are dehydrated by freeze drying.

Since spray drying of the fruit pulp did not yield free flowing powder, freeze drying of the sample was done. The resultant powder obtained was subjected to characterization for flow properties and moisture content.

4.20.3 TASTE OF THE POWDER:
The freeze dried powder of the *M. zapota* fruit pulp was tasted by human volunteers and described to be sweet to taste and retains the chicku odor.

4.20.4 FLOW PROPERTIES:
A pharmaceutical powder intended to be packed in the final packaging container is evaluated for its flow characteristics. When the powder does not flow readily, it tends to move spasmodically through the feed frame or hopper in the final container. Hence the non-uniformity in the weight and contents results in the final container. Thus the freeze dried powder was subjected to evaluation of flow properties.
The parameters for the evaluation of flow properties include the Carr"s Index, Hausner"s ratio and Angle of repose. The reference scales of the individual values are given below which helps in easy assignment of the flow characteristics.

4.20.4.1 Carr’s Index:
The freeze dried powder of the M. zapota fruit pulp exhibited a Carr"s index 28.6 hence as per the scale given below, the flow property can be described as poor.

**Scale for Carr’s Index:**

<table>
<thead>
<tr>
<th>Flow property</th>
<th>Carr&quot;s index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Good</td>
<td>11-15</td>
</tr>
<tr>
<td>Fair</td>
<td>16-20</td>
</tr>
<tr>
<td>Passable</td>
<td>21-25</td>
</tr>
<tr>
<td>Poor</td>
<td>26-31</td>
</tr>
<tr>
<td>Very poor</td>
<td>32-37</td>
</tr>
<tr>
<td>Very very poor</td>
<td>&gt; 38</td>
</tr>
</tbody>
</table>

4.20.4.2 Hausner’s Ratio:
The freeze dried powder of the M. zapota fruit pulp exhibited a Hausner"s ratio 1.41 hence as per the scale given below, the flow property can be described as poor.

**Scale for Hausner’s:**

<table>
<thead>
<tr>
<th>Flow property</th>
<th>Hausner&quot;s ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>1.00-1.11</td>
</tr>
<tr>
<td>Good</td>
<td>1.12-1.18</td>
</tr>
<tr>
<td>Fair</td>
<td>1.19-1.25</td>
</tr>
<tr>
<td>Passable</td>
<td>1.26-1.34</td>
</tr>
<tr>
<td>Poor</td>
<td>1.35-1.45</td>
</tr>
<tr>
<td>Very poor</td>
<td>1.46-1.59</td>
</tr>
<tr>
<td>Very very poor</td>
<td>&gt; 1.6</td>
</tr>
</tbody>
</table>
4.20.4.3 Angle of Repose:
The freeze dried powder of the *M. zapota* fruit pulp exhibited an Angle of repose 42° hence as per the scale given below, the flow property can be described as poor.

**Scale for Angle of Repose:**

<table>
<thead>
<tr>
<th>Flow property</th>
<th>Angle of repose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>25-30</td>
</tr>
<tr>
<td>Good</td>
<td>31-35</td>
</tr>
<tr>
<td>Fair –aid not needed</td>
<td>36-40</td>
</tr>
<tr>
<td>Passable–may hang up</td>
<td>41-45</td>
</tr>
<tr>
<td>Poor –must agitate or vibrate</td>
<td>46-55</td>
</tr>
<tr>
<td>Very poor</td>
<td>56-65</td>
</tr>
<tr>
<td>Very very poor</td>
<td>&gt; 65</td>
</tr>
</tbody>
</table>

Thus it can be concluded that the freeze dried powder did not possess very free flowing property. This could be due to the presence of mucilaginous mass in the powder. However the flow properties were improved further by addition of 0.5%w/w microcrystalline cellulose. It provided a glidant like effect slightly increased the flow parameters. Flow parameters thus obtained were Carr”s Index (24.2 - Passable, Hausner”s ratio (1.32 - Passable) and Angle of repose (38 - Fair). Usually talc or colloidal silica are incorporated as glidants in pharmaceutical powders or granules for lubrication and enhancement of flow properties however since the product is to be taken as a powder orally and needs to be dispersed in the vehicle like water or any other fluid before administration, the dispersibility of the talc and colloidal silica could be a problem hence microcrystalline cellulose which could also serve as a dispersing agent was used.

4.20.5 MOISTURE CONTENT:
The moisture content of the freeze dried powder of *M. zapota* fruit pulp was found to be less than 2%w/w. Thus the final product contains very less moisture, thereby reducing the reactive potential and render stability.
4.21 SCREENING FOR THERAPEUTIC/NUTRACEUTICAL POTENTIAL:

4.21.1 ELEMENTAL ANALYSIS:

Elemental analysis of the freeze dried powder of the fruit pulp juice was determined by dispersing it in distilled water, filtering through Whatmann filter paper and analyzed using Inductively Coupled Plasma Atomic emission Spectroscopy, at IIT Bombay. Chapter no. 5.2, Table no. 5.2.4.21.1.1 gives an elaborate list of the elements present in the *M. zapota* fruit powder.

The results revealed the composition of elements specifically copper, zinc, iron, calcium, potassium. The copper content in the human body helps in haemoglobin and collagen production, functions of the heart, energy production and absorption of iron. The zinc content in the body is essential in cell reproduction, wound healing, production of sperm and testosterone. The iron content in the body is essential for haemoglobin production, oxygen transport and energy production. The calcium content in the body is essential for maintaining bone and teeth strength, nerve, muscle and glandular function and blood clotting. While the phosphorus in the body helps in phosphorylation process, energy production, bone and teeth strength and formation of genetic material. Considering the diverse and important functions performed by the essential elements it is thus necessary that their supplementation in regular diet is must. A Chicko fruit provides all these essential elements adequately and thus should be consumed regularly. It is very important to note that the trace metals like lead and arsenic were not detected in the sample of the freeze dried pulp provided as their concentration was well below the detection limit i.e less than 0.01 ppm.

Also a comparison is made between various marketed products and the observed elemental analysis of the freeze dried powder of *M. zapota* fruit pulp providing detailed information about the nutritive value of the freeze dried product and tabulated in Chapter no. 5.2, Table no. 5.2.4.19.0 The comparison thus revealed that the freeze dried *M. zapota* fruit powder possess a high elemental composition which on consumption could help meet the daily dietary requirements.
4.21.2 ANTIOXIDANT VALUE:

Antioxidant potential of the freeze dried powder of *M. zapota* fruit pulp was determined using DPPH radical scavenging assay and total reducing power assay.

4.21.2.1 DPPH FREE RADICAL SCAVENGING ACTIVITY:

DPPH molecule contains a stable free radical. When an antioxidant able to donate an electron to DPPH is present, the typical purple color of the free DPPH radical is found to be decayed; the change in absorbance is thus measured spectrophotometrically at 517 nm. This simple test indicates information regarding the compound’s ability to donate a hydrogen atom, further not only the number of electrons a given molecule can donate, but also the mechanism of antioxidant action.

Freeze dried powder of *M. zapota* fruit pulp exhibited a fairly good DPPH scavenging activity with an IC\textsubscript{50} value as 65.54 ± 0.004 µg/ml compared to the standard ascorbic acid with an IC\textsubscript{50} value: 12.5 ± 0.004 µg/ml. The details of the experiment are tabulated in Chapter no. 5.2, Table no. 5.2.4.21.2.1.1. The results thus obtained could be due to the constituents like polyphenols, carotenoids etc. as reported by Aradhya *et al.*, 2007.

4.21.2.2 TOTAL REDUCING POWER:

The reaction between Potassium ferricyanide and ferric chloride generates potassium ferrocyanide and ferrous chloride in the presence of an antioxidant. The reducers thus present converts the Fe\textsuperscript{3+}/ferricyanide complex to the ferrous form in this method.

The total reducing power of the freeze dried powder of *M. zapota* fruit pulp was found to be comparable to the standard ascorbic acid used exhibiting a similar optical density. The observations of the experiments are tabulated in Chapter no. 5.2, Table no. 5.2.4.21.2.2.1. Thus the test sample also possesses reducing ability. The results could be attributed to the presence of antioxidants like polyphenols and Ascorbic acid contents.
4.21.3 ASCORBIC ACID CONTENT:
Ascorbic acid content however was reduced to 26.68 ± 4.5 mg/100 gm of freeze dried sample compared to the fruit pulp. The fruit pulp was obtained from the ripe *M. zapota* fruit and sent for freeze drying. The sample was freeze dried a week later due to the time required for freezing as part of the drying procedure and subjected to the further procedure of drying. The time duration for which the product was kept in the liquid state before initiation of the drying process thus led to degradation of the ascorbic acid hence the value was found to be reduced post freeze drying. This also proved the instability and degradation produced in the fresh pulp on storage in liquid state hence the research work undertaken to convert the liquid into solid form is apt and useful.

4.22 TOXICITY EVALUATION:
4.22.1 Acute Oral Toxicity:
Test substance, freeze dried *M. zapota* fruit powder was appropriately diluted with water and administered 5000 milligrams per kilograms as a single oral dose. None of the rats exhibited abnormalities in gait, fur, eyes, mucous membranes or abnormal behavior. The observation of animals in group I and group II are summarized in Chapter no. 5.2, Table no. 5.2.4.22.1.1. The Body weight is one of the important factors to be considered in Acute Oral toxicity studies as a sign of healthy animal. If the body weight changes significantly, it is considered as a first indicator of the adverse effect exhibited by the test substance frequently. Also at a dose whereby 10% or more reduction in body weight is observed in the animal, is considered toxic. At a particular dose level such reduction in body weight can be also considered as the minimum toxic dose whether it is accompanied by any other toxic effects on the body or as single observation. The change in body weights of group I and group II animals are given in Chapter no. 5.2, Table no. 5.2.4.22.1.2. and Table no. 5.2.22.1.3 respectively. The food and water consumption was also normal. Changes in body weight and food consumption in both the groups were thus found to be non-significant at the 14\textsuperscript{th} day compared to the 0 day. Various organs excised after sacrificing the animals were weighed; their organ to body weight ratios were determined and the statistical analysis using unpaired t-test was found to be non-significant of test group compared to the control group. A summary of the organ weights
and organ to body weights of Group I animals is tabulated in Chapter no. 5.2, Table no. 5.2.4.22.1.4 and Table no. 5.2.22.1.5 respectively. Similarly a summary of the organ weights and organ to body weights of Group II animals is tabulated in Chapter no. 5.2, Table no. 5.2.4.22.1.6 and Table no. 5.2.22.1.7 respectively. Further a comparison of organ to body weights of the animals in Group I and II is made and tabulated in Chapter no. 5.2, Table no. 5.2.4.22.1.8. Statistical analyses using student t test and 95% confidence limit revealed that there was no significant difference between the observed values. Hence it was concluded that the test product did not exhibit any gross toxicological signs in Acute Oral Toxicity study as per OECD guideline number 420, thus can be considered as safe to use in humans even at a high dose of 5000 mg/Kg.

4.23 STABILITY TESTING OF THE NUTRACEUTICAL PRODUCT:

In order to assign a quality standard to the product after manufacturing, the stability determination of the product is an important pre-requisite. The freeze dried powder at the end of animal studies was subjected to stability studies.

The freeze dried *M. zapota* fruit powder was found to be stable when packed in air tight container during its three months evaluation. The powder did not reveal any significant change in moisture content and pH also the ascorbic acid content was found to be 25.25 ± 2.7 mg/100 gm at the end of three months. The details of the observations are recorded in Chapter no. 5.2, Table no. 5.2.4.23.1. Thus the conversion of solid powder from the liquid slurry on freeze drying has offered stability to the *M. zapota* fruit pulp. This also indicated that the method of freeze drying is a suitable method for drying the fruit pulp. Although the overall cost of freeze drying was bit higher, the bulk of the formulation when subjected to freeze drying technique may reduce the cost of drying step. This could be possible as the small sample when subjected to drying, the quantity of nitrogen gas which is used in the procedure for a single cycle is same even if the bulk size is increased. Thus each time the sample is run, added the cost towards drying but if at one time more samples are dried then the procedure becomes cost effective.

Compilation of the overall results thus provided an extensive knowledge of the nutraceutical potential of the *M. zapota* fruits hence the wise decision of formulating solid powder product from the fruit pulp has helped to retain the nutritional contents
stable. Thus the product could be used as a nutritional supplement providing very good amounts of antioxidants, vitamin C and at the same time essential elemental load instantly on dispersing the powder in the glass of water or even fluids like milk thus fortifying the milk. Todays stressed out generation who fails to eat healthily due to lack of time or negligence such nutraceutical powder in a single spoon could energize them. Thus the present research attempted a useful venture to make the generation healthier and free of any dietary deficiencies.