CHAPTER 4
ISOLATION OF BIOACTIVE COMPOUNDS

4.1. INTRODUCTION

In 1998, Harborne has quoted that phytochemistry or plant chemistry, “is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function”.

4.1.1. Phytochemistry

Phytochemistry is the study of phyto-constituents present in all natural products. Phytochemicals are chemical compounds formed during the plant’s normal metabolic processes. These chemicals are often referred to as “secondary metabolites,” of which there are several classes including alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids (Harborne, 1973; Okwu, 2004). Phytochemicals are present in a variety of plants utilized as important components of both human and animal diets. These include fruits, seeds, herbs and vegetables (Okwu, 2005).

Natural products contain active and inactive principles in them. It is the active principles which are actually responsible for the medicinal properties of plants. The study is based on the detection, separation and isolation of the chemical constituents in plant using simpler conventional methods to complex modern sophisticated technologies. These are then applied in the field of pharmacognosy and then finally pharmacological activity is proved which is the success lying behind the cure diseases
and illness. Several new compounds are identified every day due to vast and faster methods of scientific techniques. For example, new phytochemical compounds from 12 Indian medicinal plants from the semi arid zone of Gujarat were discovered and their antibacterial activities were found to be more effective than the present day antibiotics.

Diets containing an abundance of fruits and vegetables are protective against a variety of diseases, particularly cardiovascular diseases (Okogun, 1986). Herbs and spices are accessible sources for obtaining natural antioxidants (Okwu, 2004). In addition to these substances, plants contain other chemical compounds also. These can act as agents to prevent undesirable side effects of the main active substances or to assist in the assimilation of the main substances.

4.1.2. Phenols and Polyphenols

Phenols are a member of a group of aromatic chemical compounds with weakly acidic properties and are characterized by a hydroxyl (OH) group attached directly to an aromatic ring. The simplest of phenols derived from benzene is also known as phenol and has the chemical formula C₆H₅OH. The presence of phenols is considered to be potentially toxic to the growth and development of pathogens (Okwu and Okwu, 2004). The structural classes of phenolic compounds include the polyphenols (hydrolysable and condensed tannins) and monomers such as ferulic and catechol (Okwu, 2005).

Polyphenols might interfere in several of the steps that lead to the development of malignant tumours, may play a role in inactivating carcinogens and inhibiting the expression of mutagens (Urquiaga and Leighton, 2000; Okwu, 2004). Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring.
Cinnamic and caffeic acids are common representatives of a wide group of phenyl propane derived compounds, which are in the highest oxidation state. Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two OH groups, and pyrogallol has three. The sites and number of hydroxyl groups on the phenol group to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). In addition, some authors have found that more highly oxidized phenols are more microorganisms inhibitor (Urs and Dunleavy, 1975; Scalbert, 1991,). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C-3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well characterized representative found in clove oil.

4.1.3. Flavones, Flavonoids and flavonols

Generally flavonoids can be divided into flavones, flavanols, flavanones, isoflavones and chalcones. Flavonoids usually have a three ring system consisting of a cinnamoyl based B, C-ring and a benzenoid A-ring. All three rings can be substituted with hydroxyl groups, methoxyl groups, or other substituents, leading to a large number of possible structures.

Flavonoids are a group of phytochemicals found in varying amounts in foods and medicinal plants which have been shown to exert potent antioxidant activity against the superoxide radical (Hertog et al., 1993). This may be as a result of its
antioxidant activity and subsequent inhibitions of low density lipoproteins (LDL) oxidation known to have been attributed to the dietary and supplemental intake of flavonoids and other micronutrients. Epidemiologic studies indicate an inverse relationship between intake of dietary flavonoids and coronary atherosclerotic disease (Knekt et al., 1996).

Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom. They are known to be synthesized by plants in response to microbial infection and have been found in vitro to be effective against a wide array of microorganisms (Harborne, 1973). Flavone with the molecular formula, C_{15}H_{10}O_{2}, is a commonly found plant flavonoid (Martindale, 1996). Flavonoids are potent water soluble super antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity and protect against all stages of carcinogens.

4.1.4. Thin layer Chromatography (TLC)

Of the many chromatographic methods presently available, thin layer chromatography (TLC) is widely used for the rapid analysis of drugs and drug preparation, which is a method of adsorption chromatography. This is due to the reason that the time required for elution is very short and also provides a semi quantitative information on the major active constituents of drug or drug preparation, thus enabling an assessment of drug quality and therefore for the detection of adulterations and substitutions.

4.1.5. Interactions of the compound and the adsorbent

The strength with which an organic compound binds to an adsorbent depends on the strength of the following types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole, and Van der Waals forces. With silica gel,
the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar Si-O bonds of these adsorbents and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules thus generally tend to move through the adsorbent more rapidly than the polar species.

Commercially available TLC plates (Silica gel 60 F$_{254}$ precoated TLC plates; Merck, Germany) have been used for TLC separation of active compounds. Suitable solvent systems were chosen which give sufficient separation of constituents, enough for a significant characterization of the drugs. To obtain sharply resolved zones, the quality of the material applied to the chromatogram should be as small as possible. For the detection of compounds, active constituents can be identified in both visible and ultra violet radiations as well as by using specific spraying reagents.

4.1.6. Column chromatography

Column chromatography is generally used as a purification technique; it isolates desired compounds from a mixture. In column chromatography the stationary phase, a solid adsorbent, is placed in a vertical glass column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure). The mixture to be analyzed by column chromatography is applied to the top of the column. The eluent (solvent) is passed through the column by gravity. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a
4.1.7. The solvent

The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column. Polar solvents can more effectively compete with the polar molecules of a mixture for the polar sites on the adsorbent surface and will also better solvate of the polar constituents. Consequently, a highly polar solvent will move even highly polar molecules rapidly through the column. If a solvent is too polar, movement becomes too rapid and little or no separation of the components of a mixture will result. If a solvent is not polar enough, no compounds will elute from the column. Proper choice of an eluting solvent is thus crucial to the successful application of column chromatography as a separation technique. A non-polar solvent is first used to elute a less-polar compound. Once the less-polar compound is off the column, a more-polar solvent is added to the column to elute the more-polar compound.

4.1.8. X-ray crystallography

X-ray scattering techniques are a family of non-destructive analytical techniques which reveal information about the crystallographic structure, chemical composition, and physical properties of materials and thin films. These techniques are based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy. It is a method of determining the arrangement of atoms within a crystal, in which a beam of X-rays strikes a crystal and scatters into many different directions. From the angles and intensities of these scattered beams, a crystallography can produce a ‘three-
dimensional picture of the density of electrons within the crystal’. From this electron
density, the mean positions of the atoms in the crystal can be determined, as well as
their chemical bonds, their disorder and various other information (Geerlof et al.,
2006).

4.1.9. Spectroscopy

The study of how the sample interacts with different wavelengths in a given
region of electromagnetic radiation is called spectroscopy or spectrochemical analysis.
The collection of measurement signals (absorbance) as a function of electromagnetic
radiation is called a spectrum. The mechanism of absorption energy is different in the
Ultraviolet, Infrared, and Nuclear Magnetic Resonance regions. However, the
fundamental process is the absorption of certain amount of energy (Raman, 2006).

4.1.9.1. UV-Visible spectroscopy

Chemical compounds are coloured because they absorb visible light. In
general, even organic compounds that are colourless will absorb UV light. Absorption
of UV-Visible radiation occurs via excitation of electrons from filled to unfilled
orbitals, i.e. they are electronic transitions. In the excitation, the energy of the whole
molecule increases. This overall change is typically due to promotion of a single
electron from a lower to higher energy orbital. The energy of the transition depends
on the gap between the two orbitals. Hence the molecules have characteristic
absorption spectra. In conjugated molecule, the energy separation between the orbitals
is smaller. With increasing conjugation, the decreasing energy gap is reflected by
absorption at longer wavelengths (Skoog, 1992).

4.1.9.2. FT-IR spectroscopy

Atoms within a molecule are never still. They vibrate in a variety of ways
Absorption of IR can occur if a vibrational mode is associated with a change in dipole. Functional groups have characteristic absorption frequencies. Atoms may be considered as weights connected by springs. Each vibrational mode has its own resonant frequency. If the vibrational mode involves a change in molecular dipole moment, the vibration can be induced by absorption of a photon and considered ‘IR-active’. The bigger the change in dipole, the more intense the absorption. It gives information about type of functional group present in a molecule (Bellamy, 1980). Infrared radiation spans a section of the electromagnetic spectrum having wave numbers from roughly 13,000 to 10 cm⁻¹. It is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies.

**4.1.9.3. NMR spectroscopy**

Atomic nuclei behave like small bar magnets as a result of their charge and spin. In the presence of an applied magnetic field, the spin states have different energy and the magnetic moment can align with or against the applied field. The difference in energy between the two spin states is dependent on the external magnetic field strength. It involves irradiating a sample with radio frequency radiation and resulting to the spin state separation (ΔE) will excite nuclei in the +½ state to the higher energy −½ state. The units of chemical shifting are expressed in parts per million (ppm) of the total applied magnetic field (Abraham and Loftus, 1978).

**4.1.9.3.1. ¹H NMR spectroscopy**

Just as the number of signals in the ¹H NMR spectrum tells us how many kinds of protons a molecule contains, so the positions of the signals help to tell us what kinds of protons they are: aromatic, aliphatic, primary, secondary, tertiary, benzylic,
vinyllic, acetylenic, adjacent to halogen or to other atoms or substituent groups. These different kinds of protons have different electronic environments (Bruice, 2004).

The frequency at which a proton absorbs depends on the magnetic field experienced by the proton. This effective field strength varies, depending on the environment of the proton. The environment is determined primarily by such factors as:

a) Local electron density

b) Number of nearby protons.

Each set of equivalent protons will have a unique environment, and hence will require different applied field strength in order to produce the same effective field strength, the particular field strength at which absorption occurs. At a given radio frequency (RF), all protons absorb at the same effective field strength, but they absorb at different applied field strengths. It is the applied field strength that is measured, and against which the absorption is plotted. The resulting absorption peaks give detailed information about the structure of a molecule, including:

a) **Number of signals**: Number of different equivalent protons.

b) **Positions of the signals**: Electronic environment of the proton.

c) **Intensities of the signals**: How many protons of each equivalent set.

d) **Splitting of the signal**: Environment of the proton with respect to other protons.

The natural abundance of $^1$H is 99.9844%. The relative sensitivity is 1. The chemical shift range is 0-12 ppm. It gives information about chemical environment, chemical structure, regiochemistry, stereochemistry, conformation of the molecule.
Protons in different chemical environments have different chemical shifts, $\delta$. Protons in different environments can couple to each other with a coupling constant $J$, i.e. $^1\text{H-}^1\text{H}$-spin-spin coupling. The combination of chemical shifts and coupling constants provides valuable structural information.

### 4.1.9.3.2. Chemical Shifts (Peak Position)

The variations of nuclear magnetic resonance frequencies of the same kind of nucleus, due to variations in the electron distribution, are called the chemical shift. The units of chemical shifting are expressed in parts per million (ppm) of the total applied magnetic field. The formula for the chemical shift is

$$\delta (\text{Hz}) = \frac{\text{Distance downfield from TMS (Hz)}}{\text{Operation frequency of the spectrometer (MHz)}}$$

Thus, induced magnetic fields are generated when electrons are caused to circulate in a magnetic field. Circulation of electrons about the proton itself generates a field aligned in such a way that at the proton it opposes the applied field. The field felt by the proton itself is thus diminished, and the proton is said to be **shielded**. Circulation of non-bonding pi electrons about nearby nuclei generates a field that can either oppose or reinforce the applied field at the proton, depending on the proton's location. If the induced field opposes the applied field, the proton is shielded. If the induced field reinforces the applied field, then the field felt by the proton is enhanced, and the proton is said to be **deshielded**.

The electron cloud shields the nucleus from the applied magnetic field, and electronegativity is defined as the tendency of an atom to pull electrons toward itself. Therefore, electronegative atoms remove electron density from the proton. This
causes the proton to have less electron density, and this leads to less shielding. If the proton has less shielding, it will feel the applied magnetic field more, and this leads to a higher $\Delta E$ and a higher chemical shift. Protons that are closer to the electronegative atom are in a less electron dense environment, which means that they are chemical shifts will be larger (Breitmaier, 1993).

Protons in electron dense (rich) environments sense a smaller effective magnetic field because they are more shielded by the large amount of electrons, and therefore, will require a lower frequency to come into resonance because the $\Delta E$ is smaller. The $\delta$ (ppm) will be smaller, and lower frequencies are located on the right side of the spectrum. **Upfield:** farther to the right hand side of the spectrum.

Protons in electron poor environments sense a larger effective magnetic field because they are less shielded due to fewer electrons, and therefore, they require a higher frequency to come into resonance because the $\Delta E$ is larger. The $\delta$ (ppm) will be larger, and higher frequencies are located on the left side of the spectrum. **Downfield:** farther to the left side of the spectrum.

Magnetic anisotropy is the magnetic field created by pi electrons or rings. This describes an environment where different magnetic fields are found at different points in space. Pi electrons are held less strongly than sigma electrons, so pi electrons are more able to move in response to the magnetic field. How this affects the chemical shift depends on the direction of the induced magnetic field relative to the direction of the applied magnetic field. In pi electrons found in the benzene ring and an alkene, the magnetic field induced is in the same direction as the applied magnetic field, so the protons feel a larger effective magnetic field. Therefore, the protons undergo resonance at a higher frequency due to the pi electrons. If the magnetic field induced
is oriented in the opposite direction as the applied magnetic field, the protons will feel a smaller effective magnetic field (Sakellariou et al., 2007).

4.1.9.3.3. $^{13}$C NMR spectroscopy

One way in which carbon-13 nuclear magnetic resonance ($^{13}$C-NMR or CMR) spectroscopy can be used very quickly is to determine the number of chemical shift equivalent carbons in an organic molecule. The natural abundance of $^{13}$C is 1.108%. The relative sensitivity is 1.59×10⁻². The chemical shift range is 0-200 ppm. For $^{13}$C, there is no homo nuclear coupling and separate resonance for every carbon in a molecule. It is sensitive to subtle changes in the near electronic environment but insensitive for long-range interactions (solvent effects, diamagnetic anisotropy of neighbouring groups).

4.1.9.4. Mass spectroscopy

Mass spectrometry involves the ionisation of molecules and atoms. On ionization, the molecule can break up giving fragments of different m/z ratios. Each molecule has a characteristic fragmentation pattern which can be used to identify the molecule.

It is a powerful technique for identifying unknowns, studying molecular structure, and probing the fundamental principles of chemistry and is essentially a technique for "weighing" molecules. Mass spectrometry is based upon the motion of a charged particle, called an ion, in an electric or magnetic field. The mass to charge ratio (m/z) of the ion affects this motion. Since the charge of an electron is known, the mass to charge ratio is a measurement of an ion's mass. Typical mass spectrometry research focuses on the formation of gas phase ions and the chemistry of ions. ESI (Electron Spray Ionization) is based on spraying an electrically generated fine mist of
ions into the inlet of a mass spectrometer at atmospheric pressure. This technique ionizes molecules directly from solution, so it can easily be interfaced with liquid separation methods.

The main objective of the phytochemistry in this chapter was to isolate the active phytochemicals, and characterize the isolated compounds by X-ray and spectroscopy methods.

4.2. REVIEW OF LITERATURE

New triterpenoid saponins, 1β, 2α, 3β, 19α-tetrahydroxyursolic acid 28-O-β-D-glucopyranoside, and β-sitosterol have been isolated from the stem bark of *Mimusops hexandra* and their structures have been elucidated on the basis of chemical and spectral evidence (Mala Srivastava and Singh, 1994).

The unsaponifiable matter in argan oil contains a proportion of about 20% of triterpene alcohols (Farines *et al.*, 1981). These are a complex group of plant constituents which consist mainly of five condensed cyclohexane rings with 30 carbon atoms. They can be separated from the sterols by chromatography and the few identified in crude argan oil include lupane, ursane and oleanane derivatives which include β-amyrin, butyrospermol and tirucalol as major triterpenic alcohols and represent 27.3, 18.1 and 27.9 % of the triterpene fraction, respectively.

Triterpenes and flavonoids are the main constituents of *Pouteria aublet* (Sapotaceae). Some of them have been found in regular basis in all species. Usually, triterpenes have been isolated as long chain or acetate esters. Besides long chain hydrocarbons, alcohols, acids and esters also are found mainly in species occurring in dry regions, for example, Brazilian savannah (David, 1993; Lopez, 2005; Silva, 2007). In addition, *Pouteria* species have been evaluated as enzymes sources to be used as
synthesis reagent as well as biological activity purposes (Lott and Jackes, 2001; Solis et al., 2004; Hernandez et al., 2006).

The major constituents isolated from fruits of *M. zapota* were polyphenols (methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+) catechin, (-) epicatechin, (+) gallocatechin, and gallic acid (Ma. et al., 2003). Isoaffinetin (5,7,3',4',5'-pentahydroxyflavone-6-C-glucoside) was isolated from *Manilkara indica* as a potent inhibitor of lens aldose reductase by bioassay directed fractionation.

Liu et al, (2002) reported that the study on the cytotoxic compounds from *Sarcostragus* sp., and the cyclitol derivative sarcotride-A has been isolated along with two new congeners. The gross structures of the compounds were elucidated by the aid of COSY, HMQC, and HMBC experiments. D-ononitol is present in *Simmondsia chinensis*, *Ononis spinosa*, *Medicago sativa* and *Trifolium incarnatum* (Dittrich and Brandl, 1987). D-chiro-inositol is formed from D-pinitol through demethylation, however, the enzyme catalysing its biosynthesis has not yet been characterized (Obendorf, 1997). The term cyclitol refers to polyhydroxyl cycloalkanes and their derivatives common in nature (Loewus and Loewus, 1980). Nine inositol enantiomers have been identified and distinguished by the position of the hydroxyl group (Loewus, 1990).

The antidiabetic activity guided fractionation and isolation of the 80% EtOH extracts from *Peucedani radix* (*Peucedanum japonicum*, Umbelliferae) led to the isolation and characterization of a cyclitol as active principle, that is, myo-inositol. Their structures were identified by spectroscopic methods (Lee et al., 2004). Cyclitol and pinitol have been isolated from the methanol extract of root bark of *Zantha Africana* (Beuscher et al., 1994).
4.3. MATERIALS AND METHODS

4.3.1. Glassware and chemicals

Good quality glassware and chemicals were used for all tests. All the glassware were of brand Borosil or Corning. They were washed with good detergent, rinsed in tap water and soaked in chromic acid clearing solution.

Cleaning solution (Mahadevan and Sridhar, 1996)

Potassium dichromate - 60 g
Conc. H₂SO₄ - 60 mL
Distilled water - 1 L

Potassium dichromate was dissolved in warm water, cooled and sulphuric acid was added slowly. It was mixed thoroughly and used for cleaning glassware. Then, they were rinsed thrice in tap water, finally rinsed in distilled water and dried in hot air oven. Dried glassware and media were sterilized in an autoclave for 15 min at 15 lb/sq inch pressure. These sterilization and cleaning methods were used for further experiments in the following chapters.

Chemicals

Analytical grade chemicals supplied by Hi-Media, S.D. Fine Chemicals, E.Merck, Qualigens and Sigma Chemicals (U.S.A) were used in this study.

4.3.2. Extraction

The dried powder of leaves (1 kg) of M. zapota was exhaustively extracted with methanol (72 h) at room temperature. The greenish extract was evaporated to dryness with the aid of a rotary evaporator at reduced pressure to yield a residue
called methanol crude extract (62 g). Then, it was subjected to fractionation with increasing polarity of solvents such as petroleum ether, ethyl acetate and methanol by separatory funnel and the three corresponding fractions such as petroleum ether (24.3 g), ethyl acetate (15.2 g) and methanol (20.7 g) were obtained and evaporated to dryness.

4.3.3. Qualitative phytochemical analysis

A small portion of the methanol crude dry extract of leaves of *M. zapota* was redissolved in methanol and it was used for the phytochemical tests for compounds which include phenols, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, carbohydrates and glycosides (Brain and Turner, 1975; Trease and Evans, 1989; Harborne, 1998).

4.3.3.1. Detection of alkaloids (Evans, 1997)

Solvent free extract (50 mg) was stirred with few mL of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows:

**A. Mayer’s test** (Evans, 1997)

To a few mL of filtrate, a drop or two of Mayer’s reagent was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

*Mayer’s Reagent*

Mercuric chloride (1.358 g) was dissolved in 60 mL of water and potassium chloride (5.0 g) was dissolved in 10 mL of water. The two solutions were mixed and made up to 100 mL with water.
B. Wagner’s test (Wagner, 1993)

To a few mL of filtrate, a few drops of Wagner’s reagent were added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

Wagner’s reagent

Iodine (1.27 g) and potassium iodide (2 g) were dissolved in 5 mL of water and made up to 100 mL with distilled water.

C. Hager’s test (Wagner et al., 1996)

To a few mL of the filtrate, 1 or 2 mL of Hager’s reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicated the test as positive.

D. Dragendorff’s test (Waldi, 1965)

To a few mL of filtrate, 1 or 2 mL of Dragendorff’s reagent was added. A prominent yellow precipitate indicated the test as positive.

Dragendorff’s reagent

Stock solution

Bismuth carbonate (5.2 g) and sodium iodide (4 g) were boiled for a few min with 50 mL glacial acetic acid. After 12 h, the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear, red-brown filtrate, 40 mL was mixed with 160 mL of ethyl acetate and 1 mL of water and stored in amber-coloured bottle.

Working solution

Ten mL of stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water.
4.3.3.2. Detection of carbohydrates and glycosides (Ramakrishnan et al., 1994)

The extract (100 mg) was dissolved in 5 mL of water and filtered. The filtrate was subjected to the following tests:

A. Molish’s test

To 2 mL of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 mL of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

B. Fehling’s test

One mL of filtrate was boiled on water bath with 1 mL each of Fehling solutions I and II. A red precipitate indicated the presence of sugar.

Fehling’s solution

Fehling’s solution I: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 mL with distilled water.

Fehling’s solution II: Potassium sodium tartrate (173 g) and sodium hydroxide (50 g) were dissolved in water and made up to 500 mL.

C. Barfoed’s test

To one mL of filtrate, 1 mL of reagent was added and heated on a boiling water bath for 2 min. Red precipitate indicated the presence of sugar.

Barfoed’s reagent

Copper acetate, 0.5 g was dissolved in 1.8 mL of glacial acetic acid.
D. Benedict’s test

To 0.5 mL of filtrate, 0.5 mL of Benedict’s reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic coloured precipitate indicated the presence of sugar.

Benedict’s reagent

Sodium citrate (173 g) and sodium carbonate (100 g) were dissolved in 800 mL of distilled water and boiled to make it clear. Copper sulphate (17.3 g) dissolved in 100 mL distilled water was added to it.

For detection of glycosides, 50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 h on a water bath, filtered and the hydrolysate was subjected to the following tests.

D1. Borntrager’s test (Evans, 1997)

To 2 mL of filtrate hydrolysate, 3 mL of chloroform was added and shaken. Chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides.

D2. Legal’s test

Fifty mg of the extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicated by pink colour.

4.3.3.3. Detection of saponins by foam test (Kokate, 1999)

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 15 min. A two cm layer of foam indicated the presence of saponins.
4.3.3.4 Detection of phytosterols (Finar, 1986)

Libermann-Burchard’s test

The extract (50 mg) was dissolved in 2 mL of acetic anhydride. To this, one or two drops of concentrated H₂SO₄ was added slowly along the sides of test tube. An array of colour changes showed the presence of phytosterols.

4.3.3.5 Detection of phenolic compounds and tannins

A. Ferric chloride test (Mace, 1963)

The extract (50 mg) was dissolved in 5 mL of distilled water. To this, a few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

B. Gelatin test (Evans, 1997)

The extract (50 mg) was dissolved in 5 mL of distilled water and 2 mL of 1% solution of gelatin containing 10% sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

C. Lead acetate test

The extract (50 mg) was dissolved in distilled water and to this, 3 mL of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

D. Shinoda (alkaline reagent) test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavanoids.
E. Magnesium and hydrochloric acid reduction (Harborne, 1998)

The extract (50 mg) was dissolved in 5 mL of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) were added. The presence of flavanol and its glycosides were inferred by the development of pink to crimson colour.

4.3.4. Chromatography

Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is caused to move in contact selectively with the adsorbent stationary phase.

4.3.4.1. Thin layer chromatography (Deinstrop, 2000)

In thin layer chromatography, solvent was used as the mobile phase and silica gel supported on aluminium was used as the stationary phase.

4.3.4.2. Principle

In thin layer chromatography, the stationary phase is a polar adsorbent, usually finely ground silica particles. This adsorbent is coated on a glass slide or aluminium sheet creating a thin layer of the particular stationary phase and solvents can be used as the mobile phase. The solvent is drawn through the silica gel by capillary action and the molecules are distributed by partition between the mobile and stationary phase. The partition coefficient, k, similar to the distribution coefficient for extraction, is the equilibrium constant for the distribution of molecules between the mobile phase and the stationary phase. It is this equilibrium that separates the components.

4.3.4.3. Procedure

Required piece of a precoated sheet was taken and a spot of the methanol
crude extract of *M. zapota* to be separated was placed at 0.5 cm above from the bottom of the TLC plate. Then, solvent system toluene: ethyl acetate: methanol (1: 0.9: 0.1) was used as the mobile phase. Then, the TLC plate with the spot of the extract was placed in a developing chamber, which contains the mobile phase. The liquid mobile phase is drawn up on the TLC plate and passing onto the spot of the extract by capillary action, and the solvent front moves up on the plate. As the solvent passes the on the spot, where the sample was applied, the sample began partitioning between stationary and mobile phases and the compounds separated out. The ratio of the distance traveled by a particular spot, compared to the distance traveled by the solvent front (both measured from the spot of the extract) is called the Rf value.

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\text{Distance traveled by the compound} \quad \frac{\text{Distance traveled by the solvent front}}{\text{R}_f}
\]

4.3.5. Isolation of compounds

The petroleum ether fraction and the butanol fraction were subjected to column chromatography for compound isolation.

4.3.6. Sample preparation

The semi-solid fraction of the drug was weighed and ground with small amount of silica gel, which was used for the column chromatography.

4.3.7. Column chromatography

In column chromatography, solvent was used as the mobile phase and silica gel was used as the stationary phase.
4.3.7.1. Principle

The components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. During the entire chromatography process, the eluent is collected in a series of fractions. The composition of the eluent flow can be monitored and each fraction is analyzed for dissolved compounds.

4.3.7.2. Column Packing

The silica gel was activated in hot air oven at 110°C for 1 h. The slurry of activated silica made in the hexane solvent at least 1.5 times the volume of solvent as silica, just measured out. The silica gel was thoroughly mixed by stirring vigorously to remove all the air from the silica. Before addition of the slurry a small piece of the cotton was placed at the end of the column while at the top of the knob of the column started. This is normally done by using a long stick or by a glass rod. The cotton should be compressed enough to support the column packing yet loose enough that the solvent flow will not be hindered. The cotton was placed in order to avoid slurry of the silica gel drain out while opening the knob of the column and allowed to pass through the solvent alone.

A funnel was placed on the top of the column and then, the slurry was poured into the column in small portions carefully and slowly, by keeping the knob open and with gentle taping after each addition of the slurry, in order to ensure the uniform packing. Frequently pouring of the slurry into the column was stopped and to swirl the slurry so that the silica gel was evenly mixed. A small quantity of solvent was
allowed to remain at the top of the column in order to avoid the drying or cracking of the column. Tapping is necessary to avoid the air bubble formation in the column during packing otherwise can interfere in separation. The column was run fast for some time with the hexane solvent system (low polar solvent only) in order to remove any impurities. Prepared sample was then poured into the column by using a funnel and was allowed to settle. Then, it was eluted with the mobile phase starting from non-polar such as hexane to collect fractions of 15 mL each. Each fraction was evaluated by TLC to know how many different compounds are there in it.

4.3.7.3. Petroleum ether fraction

The petroleum ether fraction (3 g) was subjected to column chromatography (17 mm column diameter) using a series of increasing polarity solvents. The sample was loaded onto the silica gel packed column in a minimum volume of 100% hexane, then eluted sequentially with 1 L of each of the following solvent systems:

- 80% hexane: 20% ethyl acetate -I
- 60% hexane: 40% ethyl acetate-II
- 40% hexane: 60% ethyl acetate-III
- 20% hexane: 80% ethyl acetate-IV
- 100% ethyl acetate-V
- 95% ethyl acetate: 5% methanol-VI
- 90% ethyl acetate: 10% methanol-VII

4.3.7.4. Butanol fraction

The butanol fraction (3 g) was subjected to column chromatography (17 mm column diameter) using a series of increasing polarity solvents. The sample was
loaded onto the silica gel packed column in a minimum volume of 100% hexane, then eluted sequentially with 1 L of each of the following solvent systems:

- 75% hexane: 25% ethyl acetate-II
- 50% hexane: 50% ethyl acetate-III
- 100% ethyl acetate-IV
- 90% ethyl acetate: 10% methanol -V
- 80% ethyl acetate: 20% methanol-VI
- 70% ethyl acetate: 30% methanol-VII
- 60% ethyl acetate: 40% methanol-VIII
- 50% ethyl acetate: 50% methanol-IX
- 40% ethyl acetate: 60% methanol-X
- 30% ethyl acetate: 70% methanol-XI

4.3.8. UV spectroscopy

The UV absorption spectra (200-400 nm) of the purified compounds (in methanol) were recorded using a Secomam S-1000 UV/Vis spectrophotometer.

4.3.9. IR spectroscopy

The IR spectra of purified compounds in KBr were recorded using a Perkin-Elmer 650 IR spectrophotometer. IR absorption positions are generally presented as wave numbers (ν). Thus, wave numbers are directly proportional to frequency, as well as the energy of the IR absorption. The wave number unit cm⁻¹ (reciprocal centimeter) is more commonly used in modern IR instruments that are linear in the cm⁻¹ scale. IR absorption information is generally presented in the form of a spectrum with wave number as the x-axis and percent transmittance as the y-axis.
4.3.10. Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra were recorded using a Bruker 300 MHz spectrometer. \(^1\)H-NMR (at 300 MHz) and \(^1\)H-\(^1\)H (COSY) and \(^1\)H-\(^{13}\)C (HETERO-COSY) correlation, DEPT 135° and \(^{13}\)C-NMR (75 MHz) spectroscopic data were collected at room temperature in d\(^6\)-DMSO and the chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) as an internal standard.

4.3.11. Mass spectroscopy

Mass spectra of the purified compounds were recorded by an electron impact (ESI) mode at 70 EV. The source, probe and scanning temperatures, which were used in this study was 0-300°C.

4.3.12. Synthesis of derivative of MZ1

The compound MZ1 was added to trifluoroacetic anhydride(CF\(_3\)CO\(_2\)O in dichloromethane in round bottom flask. The reaction mixture was kept in magnetic stirrer at room temperature for 24 h. the final products was extracted with dichloromethane and it was designated as MZ4.

4.4. RESULTS

4.4.1. QUALITATIVE PHYTOCHEMICAL ANALYSIS

Qualitative phytoconstituent analysis revealed that the presence of various phytococontituents in methanol crude extracts of leaves of Manilkara zapota (Table 4.1). The methanol crude extract of the plant showed positive results for flavonoids, phenolic compounds, saponins, alkaloids, glycosides, phytosterols and carbohydrates.
4.4.2. YIELD OF METHANOL CRUDE EXTRACT OF MANILKARA ZAPOTA

The yield of methanol crude extract was 6.2%. After fractionation, the yield of petroleum ether fraction was 2.43%, the yield of butanol fraction was 1.52% and the yield of methanol fraction was 2.07%.

4.4.3. CHARACTERIZATION OF COMPOUND MZ1

4.4.3.1. Physical characteristics

Nature : colourless crystal (Fig. 4.1).

Melting point, °C : 202±2.

Solubility : methanol and water.

Yield : 521mg.

4.4.3.2. X-ray crystallography

The x-ray crystallographic data of the compound MZ1 are given below.

Crystal data

\[ \text{C}_6\text{H}_{12}\text{O}_5 \]

\[ M_r=164.16 \]

\[ D_x=1.556 \text{ Mg m}^{-3} \]

Monoclinic, P2₁

\[ \lambda = 0.71073 \text{ Å} \]

Hall symbol \( P2_yb \)

Cell parameters from 2418 reflection

\[ a=6.4727(5) \text{ Å} \]

\[ \Theta=3.2-33.3° \]

\[ b=8.4851(6) \text{ Å} \]

\[ \mu=0.14\text{mm}^{-1} \]

\[ c=6.8249(5) \text{ Å} \]

\[ T=293 \text{ K} \]
\( \beta = 110.796(2)^\circ \)

Block, colourless

\( V = 350.41 (5) \text{ Å}^3 \)

0.21 \times 0.19 \times 0.17 \text{ mm}

\( Z = 2 \)

**Data collection**

Bruker Kappa APEX II CCD diffractometer

2418 independent reflections

Radiation source: fine-focus sealed tube

2314 reflection with \( I > 2\sigma(I) \)

Monochromator: graphite

\( R_{int} = 0.020 \)

\( T = 293 \text{ K} \)

\( \theta_{\text{max}} = 33.3^\circ \)

\( \omega = \text{scans} \)

\( \theta_{\text{min}} = 3.2^\circ \)

Absorption correction: multi-scan (SADABS, Sheldrick, 1996)

\( h = -9 \rightarrow 9 \)

\( k = -12 \rightarrow 12 \)

\( l = -9 \rightarrow 10 \)

5019 measured reflections

**Refinement**

Refinement on \( F^2 \)

Hydrogen site location: inferred neighbouring sites

Least-squares matrix: full

\( R[F2>2\sigma(F)]=0.33 \)

\( wR(F^2)=0.086 \)

\( W=1/[\sigma^2(F_0^2)+(0.0548P)^2+0.0106P] \)

Where \( P=(F_0^2+2F_c^2)/3 \)

\( (\Delta/\sigma)_{\text{max}}<0.001 \)
4.4.3.3. FT-IR spectrum

The absorption regions of the above mentioned compound MZ1 are given below. Absorption region at 3210.15 cm\(^{-1}\) suggested the presence of OH group (Fig. 4.2).

<table>
<thead>
<tr>
<th>Region, cm(^{-1})</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3316</td>
<td>O-H (str)</td>
</tr>
<tr>
<td>2931 &amp; 2851</td>
<td>C-H (str)</td>
</tr>
<tr>
<td>1419</td>
<td>C-H (def) in CH(_2)</td>
</tr>
<tr>
<td>1365</td>
<td>C-H (def)</td>
</tr>
<tr>
<td>985</td>
<td>C-O (Str)</td>
</tr>
<tr>
<td>775</td>
<td>C-H (rock)</td>
</tr>
</tbody>
</table>

4.4.3.4. Nuclear Magnetic Resonance (NMR) spectrum

i) \(^1\)H NMR spectrum

A signal ranging from \(\delta\) at 1.57-4.69 indicated that the compound MZ1 was aliphatic (Fig. 4.3). Signals were not observed in the aromatic region and therefore the compound MZ1 was confirmed to be aliphatic.

ii) \(^{13}\)C NMR spectrum

From the \(^{13}\)C NMR spectrum, it was observed that the compound MZ1 contain 6 signals (Fig. 4.4).

4.4.3.5. Chemical structure of the compound MZ1.

Based on the comparison of the physical characteristics and spectral data, the isolated compound MZ1 was confirmed to be Quercitol. This is the first report from
the leaves of *M. zapota*. The structure of MZ1 is given below:

![](image.png)

Cyclohexane-1,2,3,4,5-pentol (Quercitol).

**Molecular formula: C$_6$H$_{12}$O$_5$.**

### 4.4.4. CHARACTERIZATION OF COMPOUND MZ2

#### 4.4.4.1. Physical characteristics

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature</td>
<td>yellow powder</td>
</tr>
<tr>
<td>Melting point, °C</td>
<td>285±2.</td>
</tr>
<tr>
<td>Solubility</td>
<td>methanol and water.</td>
</tr>
<tr>
<td>Yield</td>
<td>5 mg.</td>
</tr>
<tr>
<td>Rf</td>
<td>0.69 (Fig. 4.5)</td>
</tr>
<tr>
<td>Solvent system</td>
<td>ethyl acetate : methanol (1.8 : 0.2)</td>
</tr>
<tr>
<td>UV MeOH $\lambda_{\text{max}}$ (nm)</td>
<td>226, 263, 349 (Fig. 4.6)</td>
</tr>
<tr>
<td>UV NaOMe $\lambda_{\text{max}}$ (nm)</td>
<td>267, 226 (Fig. 4.6)</td>
</tr>
</tbody>
</table>
4.4.4.2. FT-IR spectrum

The absorption regions of the compound MZ2 is given below. Absorption region at 3259.85 cm\(^{-1}\) suggested the presence of OH group (Fig. 4.7).

<table>
<thead>
<tr>
<th>Region, cm(^{-1})</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3259.85</td>
<td>O-H (Str)</td>
</tr>
<tr>
<td>2952.44</td>
<td>C-H (Str) sp(^{3})</td>
</tr>
<tr>
<td>1655.56</td>
<td>C=O (Str)</td>
</tr>
<tr>
<td>1607.56, 1607.56</td>
<td>C=C (Str) in Aromatics (ring) breathing</td>
</tr>
<tr>
<td>1500.76</td>
<td>Semicircle stretch</td>
</tr>
<tr>
<td>1455.06</td>
<td>-C-H Alkane variable bending</td>
</tr>
<tr>
<td>1338.09</td>
<td>C-O (Str) in C-O-C group</td>
</tr>
<tr>
<td>1238.04&amp;1291.74</td>
<td>O-H deformation</td>
</tr>
<tr>
<td>1013.93-1195.13</td>
<td>C-O (Str)</td>
</tr>
<tr>
<td>920.03-1195.13</td>
<td>1,2-disubstituted</td>
</tr>
<tr>
<td>674.95-1105.02</td>
<td>1,3-disubstituted</td>
</tr>
</tbody>
</table>
4.4.4.3. Nuclear Magnetic Resonance (NMR) spectrum

i) $^1$H NMR spectrum

A signal ranging from $\delta$ at 0.82-12.68 (Fig. 4.8) indicated that the compound MZ2 was aromatic. Signals were observed in the aromatic region and therefore the compound MZ2 was confirmed to be aromatic. The D$_2$O exchange spectrum also support the presence of aromatic OH groups. A signal $\delta$ at 12.96 indicated the presence of 5$^{th}$ OH group in the aromatic ring. A signal $\delta$ at 10.96 indicated the presence of 7$^{th}$ OH group in the aromatic ring. A signal $\delta$ at 9.32 indicated the presence of 4’ OH group in the aromatic ring. The resonance of 11 sugar protons as multiplet, $\delta$ at 0.82-3.97 was observed and the presence of equatorial anomic proton $\delta$ at 4.61 showed the presence of the sugar moiety.

ii) $^{13}$C NMR spectrum

From the $^{13}$C NMR spectrum, it was observed that the compound MZ2 contain 24 signals (Fig. 4.9). The values $\delta$ at 17.42, 69.93, 70.29, 70.51 and 71.17 showed the presence of rhamnose sugar moiety. The values $\delta$ at 177.7 showed the presence of carbonyl group.

4.4.4.4. MASS spectrum

The molecular weight of the compound MZ2 was found to be 499 (Fig. 4.10), which was supported by the mass spectrum.

4.4.4.5. Chemical structure of the compound MZ2.

Based on the comparison of the physical characteristics and spectral data, the isolated compound MZ2 was conformed to be flavone. This is the first report from the leaves of $M. $zapota. The structure of MZ2 is shown below:
6-(tetrahydro-3, 4, 5-trihydroxy-6-methyl-2H-pyran-2-yl)-3,5,7-trihydroxy-2-(4-hydroxy-3-(3-methylbut-2-enyl)phenyl)-4H-chromen-4-one

4.4.5. CHARACTERIZATION OF COMPOUND MZ3

4.4.5.1. Physical characteristics

- **Nature**: colourless crystal
- **Melting point, °C**: 285±2.
- **Solubility**: chloroform.
- **Yield**: 3 mg.
- **UV MeoH λmax (nm)**: 234 (Fig. 4.11)

4.4.5.2. X-ray crystallography

The x-ray crystallographic data of the compound MZ3 is given below.

<table>
<thead>
<tr>
<th>Identification code</th>
<th>MZ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C_{30}H_{50}O</td>
</tr>
<tr>
<td>Formula weight</td>
<td>426.70</td>
</tr>
<tr>
<td>Temperature</td>
<td>293(2) K</td>
</tr>
<tr>
<td><strong>Wavelength</strong></td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Crystal system, space roup</strong></td>
<td>Monoclinic, C2</td>
</tr>
</tbody>
</table>
| **Unit cell dimensions** | a = 13.5143(6) Å alpha = 90 deg.  
| | b = 6.1889(2) Å beta = 94.734(2) deg.  
| | c = 30.2305(11) Å gamma = 90 deg. |
| **Volume**           | 2519.81(17) Å³ |
| **Z, Calculated density** | 4, 1.125 Mg/m³ |
| **Absorption coefficient** | 0.065 mm⁻¹ |
| **F(000)**           | 952         |
| **Crystal size**     | 0.30 x 0.25 x 0.20 mm |
| **Theta range for data collection** | 2.70 to 26.00 deg. |
| **Limiting indices** | -16<=h<=16, -7<=k<=7, -37<=l<=37  |
| **Reflections collected / unique** | 12060 / 4894 [R(int) = 0.0280] |
| **Completeness to theta=26.00** | 99.8 % |
| **Absorption correction** | Semi-empirical from equivalents |
| **Max. and min. transmission** | 0.9871 and 0.9108 |
| **Refinement method** | Full-matrix least-squares on F² |
| **Data / restraints / parameters** | 4894 / 1 / 280 |
| **Goodness-of-fit on F²** | 1.038 |
| **Final R indices [I>2sigma(I)]** | R1 = 0.0500, wR2 = 0.1296 |
| **R indices (all data)** | R1 = 0.0590, wR2 = 0.1358 |
| **Absolute structure parameter** | 1(3) |
| **Largest diff. peak and hole** | 0.226 and -0.178 e.Å⁻³ |
4.4.5.3. Chemical structure of the compound MZ3.

(3β)-D-Friedoolean-14-en-3-ol (Taraxerol).
Molecular formula: C_{30}H_{50}O.

4.4.6. CHARACTERIZATION OF COMPOUND MZ4

4.4.6.1. Physical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature</td>
<td>colorless semisolid</td>
</tr>
<tr>
<td>Melting point, °C</td>
<td>285±2.</td>
</tr>
<tr>
<td>Solubility</td>
<td>chloroform</td>
</tr>
<tr>
<td>Yield</td>
<td>3 mg.</td>
</tr>
<tr>
<td>Rf</td>
<td>0.87 (Fig. 4.5)</td>
</tr>
<tr>
<td>Solvent system</td>
<td>chloroform: methanol (1.9: 0.1)</td>
</tr>
</tbody>
</table>

4.4.6.2. FT-IR spectrum

The absorption regions of the above mentioned compound MZ4 are given below. Absorption region at 3240.99 cm\(^{-1}\) suggested the presence of OH group (Fig. 4.12).
### Region, cm⁻¹ Vibration

<table>
<thead>
<tr>
<th>Region, cm⁻¹</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3240.99</td>
<td>O-H (str) H-bonded</td>
</tr>
<tr>
<td>2087.69</td>
<td>F-H (str)</td>
</tr>
<tr>
<td>1634.84</td>
<td>C=O (str)</td>
</tr>
<tr>
<td>1122.99</td>
<td>C-H (wag) –CH₂X</td>
</tr>
<tr>
<td>618.77</td>
<td>C-F (str)</td>
</tr>
</tbody>
</table>

#### 4.4.6.3. Nuclear Magnetic Resonance (NMR) spectrum

**i) ¹H NMR spectrum**

A signal ranging from δ at 1.28-3.70 (Fig. 4.13) indicated that the compound MZ4 was aliphatic. Signals were not observed in the aromatic region and therefore the compound MZ4 was confirmed to be aliphatic.

**ii) ¹³C NMR spectrum**

From the ¹³C NMR spectrum it was observed that the compound MZ4 contains 6 signals (Fig. 4.14).

#### 4.4.6.4. MASS spectrum

The molecular weight of the compound MZ4 was found to be 644 (Fig. 4.15), which was supported by the mass spectrum.

#### 4.4.6.5. Chemical structure of the compound MZ4.

Based on the physical characteristics and spectral data, the synthesized derivative compound MZ4 was confirmed to be **Quercitol penta-trifluoro acetate**.
This is the first report of synthesized derivative. The structure of MZ4 is given below:

\[
\begin{align*}
\text{cyclohexane-(1,2,3,4,5)-penta-trifluoro acetate (Quercitol penta-trifluoro acetate).} \\
\text{Molecular formula: C}_{16}\text{H}_{7}\text{O}_{10}\text{F}_{15}.
\end{align*}
\]

4.5. DISCUSSION

Plants are an excellent reservoir of medicines and chemical leads with which researchers can design and synthesize new drugs. In fact, about 25% of the drugs used in modern medicine owe their origins to plants from tropical rainforests. The phytochemicals can be used unmodified as drugs, as starting materials for the partial synthesis of drugs or as molecular models to synthesize new drugs. Of the more than 120 pure pharmaceutical chemicals isolated from about 100 plant species, currently in use as drugs, 40 are obtained from tropical species (Farnsworth and Soejarto, 1991).
TLC is a simple, quick, and inexpensive technique that gives the idea about the components present in the plant extract. TLC is also used to support the identity the nature of a compound in the plant extract (Graham, 1992). The separation and purification of phytoconstituents of the extract was mainly carried out using a combination of the chromatographic techniques.

In present study, for the isolation of compounds, column chromatography was used. The choice of technique depends largely upon the solubility properties of the compound to be separated. The compound MZ1 (Quercitol) was isolated in 90% ethyl acetate and 10% methanol as colourless crystals. It resembles with inositol and its derivatives. Inositols are bioactive compounds essential for regulating various physiological processes in plants and animals including human beings. Myo-inositol possesses certain vitamin like properties and is an essential growth factor for human cells grown in culture. Inositol and their derivatives play important roles as metabolites in several biological processes such as intracellular signaling and cell growth behavior (Berridge, 1993). Inositols and methyl inositols have important health benefits like free radical scavenging, detoxification, anti-cancer and anti-diabetic activities. Inositol phosphate derivatives have also involved in intracellular signal transduction pathway. Various reaction conditions have been investigated under which myo-inositol and its derivatives are regioselectively manipulated (Kiely et al., 1974). Methyl inositols are known to be present in some plant species.

The compound MZ2 (Flavone) was isolated in 30% ethyl acetate and 70% methanol as yellow powder. The compound MZ2 showed bright yellow fluorescent on TLC in ethyl acetate : methanol (1.8 : 0.2) solvent system. The Rf value of the compound was 0.69. It resembles with flavonoids. Flavonoids are a group of about 4000 naturally occuring polyphenolic compounds, found universally in foods of plant
origin (Harborne, 1986). These flavonoids display a remarkable array of biochemical and pharmacological actions viz., antiinflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities.

The compound MZ3 (Taraxerol) was isolated in 40% hexane and 60% ethyl acetate solvent ratio as colourless crystals. From the literature data, it was confirmed to be triterpene as taraxerol (Simeon et al., 2005; Jamal et al., 2009). The compound MZ4 (Quercitol trifluoro acetate) was synthesized as colourless semisolid, from the compound MZ1(Quercitol) for the confirmation of presence of OH groups in it. It is a novel compound.

4.6. SUMMARY

1. The phytochemical study of the methanol crude extract of M. zapota showed the presence of alkaloids, flavonoids, glycosides, phenolic compounds, phytosterols, carbohydrates and saponins.

2. Isolation was carried out by TLC as well as column chromatography techniques.

3. The compound MZ1 was colourless crystals and the structural elucidation was carried out by X-ray crystallography as well as FT-IR, NMR (\(^1\)H and \(^{13}\)C) and Mass spectroscopy. It was identified as Quercitol.

4. The compound MZ2 was yellow powder and the structural elucidation was carried out by UV, FT-IR, NMR (\(^1\)H and \(^{13}\)C) and Mass spectroscopy. It was identified as Flavone.

5. The compound MZ3 was colourless crystals and the structural elucidation was carried out by X-ray crystallography. It was identified as Taraxerol.
6. The MZ4, synthesized from MZ1 was a colourless semi solid compound and the structural elucidation was carried out by UV, FT-IR, NMR (1H and 13C) and Mass spectroscopy. It was identified as Quercitol penta-trifluoro acetate.

7. All the compounds are reported for the first time in M. zapota.

4.7. REFERENCES


David, V. 1993. Aplicação de técnicas cromatográficas na separação e determinação de triterpenos e hidrocarbonetos presentes nas flores, frutos e xilopódio de Pouteria torta. São Carlos, 97 p. Dissertação de Mestrado, Química, Universidade Federal de São Carlos.


Table 4.1. Qualitative phytoconstituent analysis of the methanol crude extract of *M. zapota*.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Phytoconstituents</th>
<th>Tests performed</th>
<th>Positive or negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>Steriod</td>
<td>Libermann Burchard’s test</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Flavonoids</td>
<td>Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Phenolic compounds and tannins</td>
<td>1. Ferric chloride 2. Gelatin test 3. Lead acetate</td>
<td>+ +</td>
</tr>
<tr>
<td>5.</td>
<td>Saponins</td>
<td>Foam test</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Glycosides</td>
<td>Borntager’s test</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: + - positive.
FT-IR Spectrum of compound MZ1 (Quercitol) isolated from *Manilkara zapota*
H\textsuperscript{1} NMR spectrum of compound MZ1 (Quercitol) isolated from *Manilkara zapota*
C\textsuperscript{13} NMR spectrum of compound MZ1 (Quercitol) isolated from \textit{Manilkara zapota}
Fig. 4.7

FT-IR spectrum of compound MZ2 (Flavonoid) 6-(tetrahydro-3, 4, 5-trihydroxy-6-methyl-2H-pyran-2-yl)-3,5,7-trihydroxy-2-(4-hydroxy-3-(3-methylbut-2-enyl)phenyl)-4H-chromen-4-one) isolated from *Manilkara zapota*
C\textsuperscript{13} NMR spectrum of compound MZ2 (Flavonoid) 6-((tetrahydro-3, 4, 5-trihydroxy-6-methyl-2H-pyran-2-yl)-3,5,7-trihydroxy-2-(4-hydroxy-3-(3-methylbut-2-enyl)phenyl)-4H-chromen-4-one) isolated from \textit{Manilkara zapota}
Mass spectrum of compound MZ2 (Flavonoid) 6-(tetrahydro-3, 4, 5-trihydroxy-6-methyl-2H-pyran-2-yl)-3,5,7-trihydroxy-2-(4-hydroxy-3-(3-methylbut-2-enyl)phenyl)-4H-chromen-4-one) isolated from *Manilkara zapota*
UV spectrum of compound MZ3 (Taraxerol) (3β)-D-Friedoolean-14-en-3-ol isolated from *Manilkara zapota*
FT-IR spectrum of synthesized compound MZ4 (cyclohexane-1,2,3,4,5-trifluoro acetate)
Fig. 4.13

$^{1}H$ NMR spectrum of synthesized compound MZ4 (cyclohexane-1,2,3,4,5-trifluoro acetate)
C$^{13}$ NMR spectrum of synthesized compound MZ4 (cyclohexane-1,2,3,4,5-trifluoro acetate)
Mass spectrum of synthesized compound MZ4 (cyclohexane-1,2,3,4,5-trifluoro acetate)