3. PHYTOCHEMICAL CHARACTERIZATION OF  

GISEKIA PHARNACEOIDES

3.1 INTRODUCTION

A plant contains a wide variety of chemical compounds broadly classified as primary metabolites, secondary metabolites and semantides. Primary metabolites are parts of vital metabolic pathways and most of them are of universal occurrence. Examples include starch, cellulose, carbohydrates etc. Phytochemical characterization of plant material is important as it relates to the therapeutic actions. It is perhaps obvious that different species of plant would have different chemical constituents. However these differences can extent to different varieties or even the same variety grown in different location or harvested at a different time. Different parts of plant such as leaves, bark, seeds, roots, flowers and pods can also have different active constituents.

Several groups of natural compounds mainly flavanoids (Niemann 1988), tannins (Harbone and Baxter; 2001) and piperidine alkaloids (Stermitz et al., 1994) have been recognized in some pinus species. Tannins are applied as astringents, anti-hemorragics and anti-abortifacients in veterinary medicine. Recently, studies showed that tannins can be used as an alternative to anthelmintics (Carrai et al., 2003; Paolini et al., 2003). Phytochemical studies of Harungana medagaseaiensis leaf extracts showed that it contained several components including anthracenic derivatives, flavanoids, alkaloids,
saponins, glycosides and tannins (Inuma et al., 1995; Tona et al., 1988; Olagunju et al., 2000; Okoli et al., 2002; Capasso et al., 2003) known for their antimicrobial properties (Scalbert 1991; Favel et al., 1994; Agarwal et al., 2000 a,b). Such compounds are known as secondary metabolites that are responsible for a wide variety of biological activities of the plant. Natural products are believed to be an important source of new chemical substances with potential therapeutic applicability. Therefore phytochemical evaluation of plant is essential to find out the relationship between the biological activity and the chemical structure of the biologically active phytochemicals. Their isolation, characterization and structural elucidation are an essential criterion before proceeding for its pharmacological studies. In this study the phytochemical evaluation of the major compounds were done by different chromatographic techniques like TLC, column and spectral methods like UV, IR, NMR and Mass spectroscopy.

3.2 MATERIAL AND METHODS

Chemicals

Analytical grade chemicals were obtained from Loba, Hi-media, S.D.Fine chemicals, E.Merck, Qualigens and sigma chemicals (U.S.A).

Plant powder

The dry plant material in powder form was prepared, for solvent extraction, as mentioned else where in Chapter 2.
3.2.1 Preparation of extracts

The dry powder of whole plant (5 kg) was first soaked, in Petroleum ether 40-60°C (1:4 w/v) at room temperature, for 72 h. The extract was then suction filtered using Whatmann filter paper (Harborne, 1988, 1998). This was repeated for two more times and the extracts were pooled together and concentrated at 40°C under reduced pressure using Buchi R – 153 Rota vapor. The residual plant material was extracted successively with chloroform and then methanol in the same manner as followed for petroleum ether. The crude extracts obtained from these three solvents were subjected to qualitative phytochemical screening.

3.2.2 Qualitative phytochemical screening of extracts

The different qualitative chemical tests were performed for establishing profile of given extract for its chemical composition. The following tests were performed on extracts to detect various phyto constituents present in them.

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 alkaloid test reagents as follows:

A) Mayer's test (Evans, 1997)

To a few ml of filtrate, a drop or two of Mayer's reagent were added by the side of the test tube. White or creamy precipitates if obtained indicate the test as positive.
**Preparation of Mayer's Reagent:** Mercuric chloride (1.358 g) was dissolved in 60 ml of water and potassium iodide (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, few drops of Wagner's reagent were added by the side of the test tube. A reddish-brown precipitate confirms the test as positive.

**Preparation of Wagner's reagent:** Iodine (1.27 g) and potassium iodide (2 g) was 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 filtrate, 1 to 2 ml of Hager's reagent (saturated aqueous solution of picric acid) were added. A prominent yellow precipitate indicates the test as positive.

**D) Dragendorff's test** (Waldi et al., 1965)

To a few ml of filtrate, 1 to 2 ml of Dragendorff's reagent were added. A prominent yellow precipitate indicates the test as positive.

**Preparation of 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 ethyl acetate and 1 ml water and stored in amber - coloured bottle.

**Working solution:** Ten ml stock solution was mixed with 20 ml of acetic acid and made up to 100 ml with water.

### 2. Detection of Proteins and Amino acids (Fisher, 1968; Ruthmann, 1970)

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

#### A) Millon's test (Rasch and Swift, 1960)

To 2 ml of filtrate, few drops of Millon's reagent were added. A white precipitate indicates the presence of proteins.

**Preparation of Millon's reagent:** Mercury (1g) was dissolved in 9 ml of fuming nitric acid. When the reaction was completed, equal volume of distilled water was added.

#### B) Biuret test (Gahan, 1984)

An aliquot of 2 ml of filtrate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink colours in the ethanolic layer indicate the presence of proteins.
C) Ninhydrin test (Yasuma and Ichikawa, 1953)

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to two ml of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

3. 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. Violet rings indicate the presence of carbohydrates.

B) Fehling's test

One ml of filtrate was boiled on water bath with 1 ml each of Fehling solutions A and B. Red precipitates indicate the presence of sugar.

Preparation of Fehling's solution: Fehling's Solution A: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 ml distilled water.

Fehling's Solution B: Potassium sodium tartrate (173 g) and sodium hydroxide (50 g) was dissolved in water and made up to 500 ml.
C) Barfoed's test

To 1 ml of filtrate, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 min. Red precipitate indicated presence of sugar.

Preparation of Barfoed's reagent: Copper acetate, 30.5 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. Characteristic coloured precipitates indicate the presence of sugar.

Preparation of Benedict's reagent: Sodium citrate (173 g) and sodium carbonate (100 g) were dissolved in 800 ml 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.

E) Borntrager's test (Evans, 1997)

To 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colours indicate the presence of glycosides.
**F) Legal's test**

The extract 50 mg was dissolved in pyridine; sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicating by pink colour.

4. **Detection of Fixed oils and Fats** (Kokate, 1999)

   **A) Spot test**
   
   A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil.

   **B) Saponification test**
   
   A few drops of 0.5 N alcoholic potassium hydroxide solution was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 h. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

5. **Detection of Phenolic compounds and Flavonoids**

   **A) Ferric chloride test** (Mace, 1963)
   
   The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. Dark green colours indicate 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 precipitates indicate the presence of phenolic compounds.
**C) Lead acetate test** (Evans, 1997)

The extract (50 mg) was dissolved in distilled water and to this; 3 ml of 10% lead acetate solution was added. Bulky white precipitates indicate the presence of phenolic compounds.

**D) Alkaline reagent test** (Evans, 1997)

An aqueous solution of the extract was treated with 10 % ammonium hydroxide solution yellow fluorescence indicates the presence of flavonoids.

**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. Formation of pink to crimson colour, if any, was inferred by the presence of flavonol glycosides.

6. **Detection of Phytosterols** (Finar, 1986)

**Libermann-Burchard's test**

The extract (50 mg) was dissolved in 2 ml acetic anhydride. To this, one or two drops of concentrated sulphuric acid were added slowly along the sides of the test tube. An array of colour changes if any shows the presence of phytosterols.

7. **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 2 cm layer of foam indicates the presence of saponins.
8. **Detection of Volatile oil** (James *et al*., 1996)

   In a volatile oil estimation apparatus, 50 g of powdered plant material was taken and subjected to hydro-distillation. The distillate was collected in a graduated tube of the assembly, where in the aqueous portion automatically separated out from the volatile oil.

9. **Detection of Gum and Mucilage’s** (Whistler and Miller, 1993)

   The extract (100 mg) was dissolved in 10 ml of distilled water and to this, 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitates indicate the presence of gums and mucilage’s.

10. **Detection of Tannins**

    The extract was mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of tannins.

**Separation by column chromatography**

    Each of the three extracts (concentrates) obtained from successively by using petroleum ether, chloroform and methanol was subjected to column chromatography to separate the active principles. A glass column, measuring 90 x 3.5 cm, was packed with silica gel (100-200 mesh) in slurry form in the petroleum ether, benzene, ethyl acetate, chloroform and methanol solvent mixture. Concentrated extract was ground well with a small amount of silica gel and loaded on to the top of the column that was eluted in succession with solvents of increasing polarity (petroleum ether, benzene, ethyl acetate,
chloroform, and methanol respectively) and 20 ml of each fraction were collected as they came off the column.

**Detection by TLC**

Column chromatographic fractions eluted at different intervals were revolved on a TLC plate. Fractions showing similarity in band pattern were pooled together and concentrated in *vacuo* for further characterization studies, after confirmation by TLC using appropriate mobile phase. TLC plates were made by using a homogenous suspension of silica gel prepared by mixing 40 g of silica gel G 200 mesh in about 85 ml distilled water. The suspension was then poured into stahl TLC (UND PLAN model) spreader, which was adjusted to 0.25 mm thickness. Carrier plates (20 cm x 5 cm) of the same thickness were laid in a row on a template and coated in a single passage of the spreader over them. These plates were left on the template for air drying the transparency of the layer disappeared and dried at 110°C for 30 min and kept in desiccators. The crude extracts separate compounds were spotted at 2 cm from the edge of the TLC plate. The chromatogram was developed in a mixture of suitable solvent system and dried at room temperature. The spots were visualized with UV light at 254 or 346 nm. The dried TLC plates were then sprayed with 10% H₂SO₄ and heated at 110°C for 5 min (Waldi, 1965). Alternatively, the developed TLC plates were placed in iodine chamber (Harborne, 1998). The Rₚ values of the coloured spots were recorded.
3.2.3 Characterization of isolated compounds

UV and Visible Spectroscopy

UV and Visible spectral analysis of the purified sample either in chloroform or in methanol, depending upon the solubility of the compound, was done by using Perkin Elmer Lambda 25 UV-Visible Spectrometer at a wavelength range of 200 – 800 nm.

Infra Red Spectroscopy

To determine the chemical functional groups in the purified samples, Infra Red (IR) spectra of samples were recorded in Perkin Elmer RX – I – FT-IR spectrometer in the range of wave number 500 to 4000 cm⁻¹ using KBr pellet, and operating in the transmittance mode.

¹H- NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectral studies were done with the purified compounds using JOEL–500 MHz NMR spectrometer. CDCl₃ (Deuterochloroform) and TMS (Tetramethyl silane) were used as solvent and as internal standard respectively. Chemical shifts were measured in δ scale in ppm.

¹³C-NMR Spectroscopy

Nuclear magnetic resonance spectral data of purified compounds were obtained using JOEL –125 MHz NMR Spectrometer. CDCl₃ (Deuterochloroform) and TMS (Tetra methyl silane) were used as solvent and as internal standard respectively. Chemical shifts were measured in δ scale in ppm.
LC – MS Spectroscopy

Liquid chromatography combined with mass spectrum of the isolated compound in pure form was recorded on LC – MS 2010A instrument at 70ev by direct inlet method.

**SCHEME- I**

Dried powder of whole plant of *Gisekia pharnaceoides* (5kg)

- Macerated with petroleum ether (40-60°C) 7 liters for 72 h
- Macerated with Chloroform 6 liters for 72 h

**Petroleum ether extracts (GPP)**

- 150.7g (3%)
- Column chromatography on silica gel (100-200 μ meshes size). Successive elution with petroleum ether, benzene, ethyl acetate, chloroform, methanol (Increasing polarity).
- CD – 1, 200 mg; Melting point 68°C
- Resolved on silica gel-Petroleum ether: Benzene (1:1) TLC system

**Chloroform extracts**

- 140g (2.8%)
- Macerated with Methanol

**Methanol extracts (GPM)**

- 250 g (5%)
- Column chromatography on silica gel (100-200 μ meshes size). Successive elution with petroleum ether, benzene, ethyl acetate, chloroform, methanol (Increasing polarity).
- No yield was obtained
- Resolved silica gel-Chloroform: Methanol (1:9) TLC system

CD – 2, 500 mg; Melting point 278°C
3.3 RESULTS

Preparation of extracts

The dried whole plant powder (5 kg) of *Gisekia pharnaceoides* was subjected to successive extraction in the order of petroleum ether, chloroform and methanol solvents as mentioned in scheme I. The extracts obtained in this study were named as petroleum ether extract (GPP), chloroform extract (GPC) and methanolic extract (GPM). The yield of GPP, GPC and GPM were found to be 3%, 2.8% and 5% respectively. All the extracts were subjected to qualitative phytochemical screening and column chromatography followed by TLC.

Qualitative phytochemical screening

Qualitative chemical test revealed the presence of various phytochemicals in petroleum ether, chloroform and methanol extracts of *Gisekia pharnaceoides* (Table.3). Petroleum ether extracts showed positive test for carbohydrate and glycosides, phenolic compounds and flavonoids, gums and mucilage and the tannins. Chloroform extract showed positive test for carbohydrate and glycosides, phenolic compound and flavonoids and tannins. Whereas methanolic extract was shown to contain carbohydrate and glycosides, phenolic compounds and flavonoids, proteins and amino acids and the tannins.

Yield of pure compound

Two compounds were isolated in a detectable size on TLC plate, from the extracts by column chromatographic methods. Compound 1 isolated from
GPP and the compound 2 isolated from GMP are designated as CD-1 (yield-200mg, white colour) and CD-2 (yield-500mg, yellow colour) respectively.

**Characterization of CD-1**

The characterization of the CD-1 and elucidation of its structure were carried out using various spectroscopic techniques such as UV, IR, $^1$H-NMR, $^{13}$C-NMR and LC-MS.

**(i) Physical characteristics**

The CD-1 (isolated from GPP) was white in colour and the melting point was found to be 68°C. It showed an $R_f$ value 0.62 on TLC plate in the solvent system of petroleum ether: benzene (1:1). The spot turned light green colour after spraying with 10% (v/v) H$_2$SO$_4$ and heating in hot air oven at 110°C for 10 min. it was blue in colour in presence UV light (short UV at 254 nm). It gave no blue colour with ferric chloride indicating the absence of phenolic hydroxyl group. The compound was soluble in chloroform and acetone.

**(ii) UV –Vis spectrum**

The UV –Vis spectrum of CD-1 exhibited no absorption maximum in the spectrum, recorded at 200-800 nm range (data not presented), implied the absence of unsaturation in the compound studied.
(iii) IR spectrum

Fig.14 shows the IR spectrum of CD-1 and displayed peaks at different wave numbers viz. 2918 cm\(^{-1}\), 2833 cm\(^{-1}\), 2356 cm\(^{-1}\), 1466 cm\(^{-1}\), 1337 cm\(^{-1}\) and 722 cm\(^{-1}\) respectively.

Methyl C-H stretching absorption were observed at 2918 cm\(^{-1}\), and an absorption at 2833 cm\(^{-1}\) represents the CH\(_2\) stretching vibration and the aliphatic C-H of hydrocarbons. The bending vibration for CH\(_2\) and CH\(_3\) were seen at 1466 cm\(^{-1}\) and 1337 cm\(^{-1}\) respectively. Long chain band was observed at 722 cm\(^{-1}\).

(iv) \(^1\)H-NMR spectrum

\(^1\)H-NMR spectrum of CD-1 (Fig.15) revealed the following details:
\(^1\)H-NMR (500MHz, CDCl\(_3\)) \(\delta\) in ppm: 0.87 (brS, 6H), 1.24 (brS, 56H) and 1.57 (brS, 4H).

Terminal methyl groups were observed as broad singlet at \(\delta\) 0.87 ppm. The methylene groups adjacent to terminal methyl groups were observed as a broad singlet at \(\delta\) 1.57 ppm. The other methylene protons gave an intense broad singlet at \(\delta\) 1.24 ppm. The detailed \(^1\)H-NMR spectral study indicated the presence of terminal methyl group.

(v) \(^{13}\)C-NMR spectrum

The structure proposed for CD-1 is further confirmed from a study of its \(^{13}\)C-NMR spectrum (Fig.16 and 17). \(^{13}\)C-NMR (125MHz) \(\delta\) in ppm: 14.22, 22.78, 29.46, 29.79 and 32.02.
In the $^{13}$C-NMR spectrum, terminal methyl groups were observed at $\delta$ 14.22 ppm. The subsequent methylene groups were observed at $\delta$ 22.78, 29.46 and 32.02 ppm respectively. The other methylene groups gave an intense signal at $\delta$ 29.79 ppm.

(vi) LC-MS spectrum

In combined Liquid Chromatography – Mass Spectrum, LC chromatogram of compound CD-1 (Fig.18) showed single peak revealed that the compound CD-1 is a single pure compound. The mass spectrum of the compound CD-1 (Fig.18) showed $M^+$ at m/z 450; Lose of C$_2$H$_5$, C$_3$H$_7$, C$_4$H$_9$, C$_5$H$_{11}$, C$_6$H$_{13}$, C$_7$H$_{15}$, C$_8$H$_{17}$, C$_9$H$_{19}$ & C$_{10}$H$_{21}$ groups gave peak at 420, 407, 392, 379, 365, 351, 337, 323 & 309 respectively. The peak at 296, 281, 267, 253, 239, 225, 211, 197, 183 and 169 were due to loss of C$_{11}$H$_{23}$, C$_{12}$H$_{25}$, C$_{13}$H$_{27}$, C$_{14}$H$_{29}$, C$_{15}$H$_{31}$, C$_{16}$H$_{33}$, C$_{17}$H$_{35}$, C$_{18}$H$_{37}$, and C$_{19}$H$_{39}$ & C$_{20}$H$_{41}$ respectively. Other major peaks appeared at m/z 155, 141, 127, 113, 99, 85, 71, 57 & 43 respectively.

Based on the physical characteristics and spectral analysis the structure of the CD-1 was elucidated.

Characterization of CD-2

The CD-2 was isolated from the crude methanolic extract of *Gisekia pharnaceoides*, the characterization of the compound and elucidation of its structure were carried out using various spectroscopic techniques such as UV, IR, $^1$H-NMR, $^{13}$C-NMR and LC-MS.
(i) Physical characteristics

The CD-2 appeared yellow in colour and the melting point was found to be 278°C. It showed an Rf value 0.67 on TLC plate in the solvent system of chloroform: methanol (9:1). The spot turned light green colour after spraying with 10% (v/v) H2SO4 and heating in hot air oven at 110°C for 10 min. It was yellow in the presence of UV light at 254 nm and produced a blue colour with ferric chloride, indicating the presence of phenolic hydroxyl group. The compound was soluble in methanol and acetone.

(ii) UV –Vis spectrum

The UV –Vis spectrum of CD-2 (Fig.19) recorded at 200 -800 nm wavelength range exhibited a maximum absorption at 328 nm, indicates the presence of chromophores in the compound studied.

(iii) IR spectrum

The IR spectrum of CD-2 (Fig.20) showed absorption peaks at 3345 cm⁻¹, 2361 cm⁻¹, 1660 cm⁻¹, 1614 cm⁻¹, 1504 cm⁻¹, 1380 cm⁻¹, 1308 cm⁻¹, 1251 cm⁻¹, 1176 cm⁻¹, 1009 cm⁻¹, 974 cm⁻¹, 818 cm⁻¹, 722 cm⁻¹ and 660 cm⁻¹ respectively.

Hydroxyl or phenolic O-H absorption were observed at 3345 cm⁻¹, broad O-H stretching absorption were observed at 2361 cm⁻¹, C=C stretching band of vinyl group, conjugation with phenyl group absorptions were observed at 1660 cm⁻¹ and 1614 cm⁻¹ respectively. C-O-H bending were
observed at 1380 cm$^{-1}$, C-O-H in plane bending were observed at 1308 cm$^{-1}$, C-O stretching absorption was observed at 1251 cm$^{-1}$, C-O-C stretching band absorption were observed at 1176 cm$^{-1}$, $=\text{C-H}$ wagging absorption were observed at 1009 cm$^{-1}$ and 974 cm$^{-1}$ respectively. Out of plane C-H bending absorption was observed at 818 cm$^{-1}$ and broad hydroxyl bond out of plane O-H bending absorption were observed at 660 cm$^{-1}$.

(iv) $^1\text{H-NMR spectrum}$

$^1\text{H-NMR}$ spectrum of CD-2 (Fig.21) revealed the following details:

$^1\text{H-NMR}$ (300MHz, CDCl$_3$) $\delta$: 6.19 (d, $J=1.5$Hz, 1H), 6.44 (d, $J=1.2$Hz, 1H), 6.91 (d, $J=6.6$Hz, 2H), 8.03(d, $J=6.6$Hz, 2H), 9.42(br S, 1H), 10.19(br S, 1H), 11.00(br S, 1H) and 12.48( S, 1H).

The hydroxyl protons were observed as broad singlet at $\delta$ 9.42, 10.19 and 11.00 ppm respectively. The aromatic protons were observed in the region at $\delta$ 6.19 – 12.48 ppm.

The detailed $^1\text{H-NMR}$ spectral study indicated the presence aromatic protons. The hydroxyl protons were observed as broad singlet at $\delta$ 9.42, 10.19 and 11.00 ppm respectively. The aromatic protons were observed in the region at $\delta$ 6.19 – 12.48 ppm. The integration value of the NMR for hydrogen accounted for 10 protons as evidenced by mass spectrum (Fig.24).

(v) $^{13}\text{C-NMR spectrum}$

The structure proposed for CD-2 was further substantiated confirmed from the data of its $^{13}\text{C-NMR}$ spectrum (Fig.22 and 23).
\[^{13}\text{C-NMR (75MHz)}\ \delta \text{ in ppm: 93.95, 96.67, 103.50, 115.90, 122.14, 129.97, 136.10, 147.27, 136.64, 139.68, 167.73, 169.33 and 176.33.}\]

Aromatic and olefinic carbons were observed in the region \(\delta 93.95 – 164.33\) ppm. The carboxyl carbon was observed at \(\delta 176.33\) ppm.

*(vi) LC-MS spectrum*

In combined Liquid Chromatography–Mass (LC-MS) Spectrum, LC chromatogram of CD-2 (Fig.24) showed single peak suggesting that the compound is single and pure. The mass spectrum of CD-2 (Fig. 24) showed \(M^+\) at \(m/z\) 286.

Based upon the physical characteristics and spectral analysis data, the structure of the CD-2 was elucidated.

**3.4 DISCUSSION**

The phytochemical screening of the crude extract of *Gisekia pharnaceoides* obtained petroleum ether; chloroform and methanol in succession reveal the presence of different group of compounds of biologically important. For instant, the presence of Phenolic compounds, as detected (Table.3), suggests that these compounds may exhibit anti microbial property as has been reported earlier Ofokansi *et al* (2005). Plants such as *B. pinnatum* containing phenolic compound have been reported to be effective in the treatment of typhoid fever and other bacterial infections, particularly those caused by *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,*
*Pseudomonas aeruginosa, klebsiella aerogenes, klebsiella pneumoniae* and *Salmonella typhi* Ofokansi *et al.*, (2005). The phenolic compound containing *B. pinnatum* has also been reported to be used in treating the placenta and navel wounds of newborn babies in Nigeria (Okwu 2004), may be to enhance healing by controlling the infection. These properties of phenolic compounds bestow high medicinal activities on the extracts from *Gisekia pharnaceoides* as well. Apart from phenolic compounds, other secondary metabolite constituents of *Gisekia pharnaceoides* detected in the crude extracts include the alkaloids and flavonoids. Alkaloids, isolated in pure form and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects (Stray, 1998; Okwu and Okwu, 2004). They exhibit marked physiological activity when administered to animals. Another type of compounds, tannins has stringent properties, hastens the healing of wounds and inflamed mucous membranes. These perhaps, explain why traditional medicine healers in Africa and India often use *Gisekia pharnaceoides* in treating wounds and burns (Musa *et al.*, 2006). Flavonoids, on the other hand are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity (Salah *et al.*, 1995; Del-Rio *et al.*, 1997; Okwu, 2004). Flavonoids in intestinal tract lower the risk of heart disease. As antioxidants, flavonoids from these plants provide anti-inflammatory activity (Okwu, 2004). Therefore the flavonoid rich (0.2% w/w) *Gisekia pharnaceoides* (as mentioned in scheme I), can also be used to prevent the oxidative insult and the resultant metabolic disorder in the cells.
CD-1 obtained as a pure compound from the column chromatography and detected by TLC using petroleum ether: benzene (1:1) as mobile phase, was white colour and the melting point was found to be 68°C. It was found to be soluble in chloroform. The purity of CD-1 was further confirmed with liquid chromatography spectrum that showed a peak of 100% purity. The result of the mass spectrum revealed that the molecular weight of the compound was 450.

The IR spectrum of CD-1 showed peak at 2918 cm⁻¹, 2833 cm⁻¹, 2356 cm⁻¹, 1466 cm⁻¹, 1337 cm⁻¹ and 722 cm⁻¹. Which are characteristics of long chain alkanes. The detailed ¹H-NMR spectral study indicate the presence of terminal methyl group which are observed as broad singlet at δ 0.87 ppm. The methylene groups adjacent to terminal methyl groups are observed as a broad singlet at δ 1.57 ppm. The other methylene protons gave an intense broad singlet at δ 1.24 ppm.

The integration value of the NMR for hydrogen accounted for 66 protons, as evidenced by mass spectrum. The ¹³C-NMR spectral data provided an additional support to deduce the CD-1. In the ¹³C-NMR spectra, terminal methyl groups are observed at δ14.22 ppm. The subsequent methylene groups are observed at δ 22.78, 29.46 and 32.02 ppm respectively. The other methylene groups displayed an intense signal at δ 29.79 ppm.

Based on these results, the structure CD-1 was derived, and it was identical to that of dotriacontane (Heilbron 1953; Greenway et al., 1953). All the data clearly supported the confirmation of compound CD-1 to be dotriacontane with the following structure.
CD-2 obtained as a pure compound from the column chromatography and detected by TLC using chloroform: methanol (1:9) as mobile phase, was yellow in colour and the melting point was found to be 278°C. It showed an R<sub>f</sub> value 0.67 on TLC plate. It formed blue colour with ferric chloride indicating the presence of phenolic hydroxyl group. The compound was soluble in methanol and acetone. The purity of CD-2 was further confirmed using the results of liquid chromatography spectrum which showed a peak of 100% purity. The mass spectrum of the compound revealed the molecular weight of 286.

IR spectrum of CD-2 showed the absorption peak at 3345 cm<sup>-1</sup> represent the free hydroxyl group of alcohols or phenols. Absorption at 2361 cm<sup>-1</sup> and 660 cm<sup>-1</sup> represent the OH bending respectively. The presence of C=C stretching of vinyl group and its conjugation with phenyl group absorb at 1660 cm<sup>-1</sup>, 1614 cm<sup>-1</sup> respectively. The presence of C-O-H bending is seen by an absorption at 1380 cm<sup>-1</sup> where the C-OH in plane bending at 1308 cm<sup>-1</sup>. The peaks at 1251 cm<sup>-1</sup> and 1176 cm<sup>-1</sup> indicate the presence of C-O stretching and C-O-C stretching respectively. The two bands arising from =C-H wagging in aromatic alkenes occur near 1009 cm<sup>-1</sup>, 974 cm<sup>-1</sup> respectively.
The in phase, out of – plane bending of a hydrogen atom is strongly coupled to adjacent hydrogen atoms. The position of absorption of the out-of-plane bending band appeared at 818 cm\(^{-1}\) is therefore characteristic of the number of adjacent hydrogen atoms on the ring. The bands are frequently intense and appear at 900- 675 cm\(^{-1}\).

The detailed \(^1\)H-NMR spectral study indicated the presence aromatic protons. The hydroxyl protons were observed as broad singlet at \(\delta\) 9.42, 10.19 and 11.00 ppm respectively. The aromatic protons were observed in the region at \(\delta\) 6.19 – 12.48 ppm. The integration value of the NMR for hydrogen accounted for 10 protons as evidenced by mass spectrum. The \(^{13}\)C-NMR spectral data provided an additional support to deduce the CD-2. In the \(^{13}\)C-NMR spectra, aromatic and olefinic carbons were observed in the region \(\delta\) 93.95 – 164.33 ppm. The carboxyl carbon was observed at \(\delta\) 176.33 ppm.

The identity of the compound CD-2 was finally confirmed by comparison with the reported data (Brough 1981). All the data clearly supported the confirmation of compound CD-2 to be kaempferol with the following structure.
REFERENCE


