Investigation of Flavonoidal Content and Biological Activities of *Chorisia Insignis* Hbk. Leaves

1Taha S. El Alfy, 2Salma A. El Sawi, 3Amany Sleem and 2Doaa M. Moawad

1Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.  
2Pharmacognosy and 3Pharmacology Departments, National Research Centre, Dokki 12622, Cairo, Egypt.

Abstract: Leaves of *Chorisia insignis* HBK. (Family Bombacaceae) were studied for their flavonoidal content. The *n*-butanol fraction of the 70% ethanol extract was subjected to different chromatographic methods (column chromatography, preparative paper chromatography and/or preparative thin layer chromatography). Three flavonoids were isolated for the first time from the genus *Chorisia*: Rutin, Luteolin-7-O-β-D-rutinoside and Apigenin-7-O-β-D-rutinoside. Their chemical structures were established using UV, 1H-NMR, 13C-NMR and ESI-MS spectral data as well as acid hydrolysis. The LD₅₀ of the total and the aqueous extracts and the successive fractions of the 70% ethanol extract revealed low toxicity of the plant. All the extracts showed significant anti-inflammatory activities against carrageenan-induced rat paw oedema as compared with indomethacin. Significant antihyperglycemic activities were exhibited by the total and the aqueous extracts and the ethyl acetate fraction in alloxan-induced diabetic rats as compared with metformin. The total and the aqueous extracts and the ethyl acetate and *n*-butanol fractions showed significant antioxidant activities as indicated by the rise in blood glutathione in diabetic rats as compared with vitamin E. Pretreatment of rats with the total extract, the aqueous extract or the ethyl acetate fraction for one month before and after liver damage, induced by intraperitoneal injection of 5 ml/kg of 25% CCl₄ in liquid paraffin, resulted in significant decrease in AST, ALT and ALP as compared with silymarin, revealing significant hepatoprotective activities of these extracts.

Key words: Antihyperglycemic, Anti-inflammatory, Antioxidant, Apigenin, *Chorisia insignis*, Flavonoids, Hepatoprotective, Luteolin, Rutin.

INTRODUCTION

There are many reasons for the current increased use of herbal medicines. These may range from the appeal of products from "nature" and the perception that such products are "safe", to more complex reasons related to the philosophical views and religious believes of individuals (Heinrich et al., 2004).

*Chorisia insignis* HBK., vernacularly known as white floss silk tree, belongs to family Bombacaceae. It is native to South America, Peru, Brazil and Argentina (Huxley, 1992; Bailey, 1976; Barwick, 2004). *Chorisia* was named in honor of the botanical artist and traveler Ludwig I. Choris (1795–1828, 19th century) (Huxley, 1992).

*Chorisia insignis* is mainly cultivated for its ornamental brilliant flowers. It is also cultivated for the silky white fiber (or floss) that is obtained from the ripened seeds. This floss has been used to stuff cushions, pillows and vests which explain the common name of this tree, "floss silk tree" (Huxley, 1992; Bailey, 1976). Phytochemical investigations of *C. insignis* remained confined to the isolation of one flavonoid (rhoifolin glycoside) from fresh leaves (Coussio, 1965). Thus, it was deemed of interest to investigate the phytoconstituents as well as the anti-inflammatory, antihyperglycemic, antioxidant and hepatoprotective activities of the plant.

MATERIAL AND METHODS

Plant Material: Samples of the leaves of *C. insignis* HBK. were collected from National Research Centre garden, Dokki, Egypt in June 2006, and were kindly authenticated by Dr. Mohamed Gibali, Senior Botanist, Botany

Corresponding Author: Taha S. El Alfy, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

1334
Department, National Research Centre (NRC) and by Dr. Tereez Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of Orman Botanical Garden, Giza, Egypt. A voucher specimen (no. 23569) is kept at CAIRC (National Research Centre Herbarium). The collected leaves of the plant under investigation were air dried and kept in tightly-closed containers.

**Authentic Reference:**

**Flavonoid:**
Luteolin, apigenin and quercetin were available in the department of Phytochemistry, National Research Centre.

**Sugars:**
Glucose, galactose, rhamnose, xylose, arabinose, ribose, fructose, glucuronic acid and galacturonic acid (Sigma).

**Adsorbents for Chromatography:**
- Silica gel 60 F254 for Thin Layer Chromatography (TLC) (Fluka Chemie AG, Switzerland).
- Cellulose powder for Column Chromatography (CC) (Fluka, Sigma-Aldrich Chemie GmbH, Germany). Sephadex LH-20 for CC (Fluka Chemie AG, Switzerland).
- Polyamide 6 for CC (Sigma-Aldrich Chemie GmbH, Germany).
- Sheets of Whatmann filter paper (3MM) for Preparative Paper Chromatography (PPC) (Whatmann Ltd., Maidstone, England).

**Solvent Systems (v/v):**

\[ S_1: \text{n-BuOH: Acetic acid: } H_2O \ (4:1:5) \]
\[ S_2: \text{Distilled } H_2O \]
\[ S_3: \text{n-BuOH saturated with } H_2O \]
\[ S_4: \text{MeOH: } H_2O \ (10:90) \]
\[ S_5: \text{MeOH (100\%)} \]
\[ S_6: \text{CH}_2\text{Cl: MeOH: } H_2O \ (7:3.5:0.5) \]
\[ S_7: \text{Acetic acid: } H_2O \ (15:85) \]
\[ S_8: \text{Phenol: } H_2O \ (80:20) \]

**Experimental Animals:**
Adult male albino rats of Sprague Dawely Strain of 130-150 g b.wt. and albino mice of 25-30 g b.wt. were used for the study of the crude extracts. Medical Research Ethical Committee (MREC) in the National Research Centre has approved the project (09/118). The animals were obtained from the animal house colony of National Research Centre, Dokki, Egypt and were kept under the same hygienic conditions and on a standard laboratory diet.

**Animals Diet:**
It consisted of vitamin mixture (1\%), mineral mixture (4\%), corn oil (10\%), sucrose (20\%), cellulose (0.2\%), casein-95% pure (10.5\%) and starch (54.3\%).

**Drugs:**
- Indomethacin (Epico, Egyptian Int. Pharmaceutical industries Co, A.R.E., under license of MERK & Co. INC-RAHAWY, N.J., USA) was used as a standard anti-inflammatory drug.
- Carrageenan (Sigma Co) was used for the induction of acute inflammation in rats.
- Alloxan (Sigma Co) was used for the induction of diabetes in rats.
- Metformin (Cidophage)® (Chemical Industries Development (CID), Giza, A.R.E) was used as a standard anti-diabetic drug.
- Vitamin E (dl- \alpha-\text{tocopheryl acetate}) (Pharco Pharmaceutical Co). It is available in the form of gelatinous capsules; each contains 400 mg vitamin E.
- Silymarin (SEDICO, Cairo, A.R.E) used as a standard hepatoprotective drug.
- Doses of the drugs were calculated according to Paget and Barner's (1964), and were administered orally by gastric tube.
Biochemical Kits and Reagents:
- Carbon tetrachloride (CCl₄) (Analar).
- 1,1-diphenyl-2-picrylhydrazil (DPPH) (Sigma® Chemical Company (EUA)).
- Biodiagnostic kits used for the assessment of blood glucose level, serum AST, ALT and ALP and blood glutathione (QCA, Spain).

Apparatus:
- UV-Visible Spectrophotometer: UV-VIS double beam UVD-3500 spectrophotometer, Labomed, Inc. spectrophotometry.
- NMR Spectroscopy: Typical conditions of NMR spectrometer with spectral width 500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR (Joel ECA 500).
- Electrospray Ionization Mass Spectrometer: ESI-MS, Thermo Finnigan (ion trap).
- Electro Spin Resonance (ESR) spectroscopy: Brucker, ELEXSYS, E 500, Germany.

Preparation of Crude Extracts:
Successive Extracts:
550 g of air-dried powdered leaves were exhaustively defatted using petroleum ether (40-60°C) (E1), then extracted by refluxing with 70% ethanol. The ethanol extract was combined and evaporated under reduced pressure to dryness to give 145 g. The dry residue was then suspended in water (600 mL) and partitioned successively with ether (E2) (10×100 mL) followed by chloroform (E3) (15×100 mL), ethyl acetate (E4) (15×100 mL) and n-butanol (E5) (12×100 mL). The solvents were evaporated to dryness under reduced pressure at 40°C.

Total Extract (E6):
100 g of air-dried powdered leaves were exhaustively extracted by refluxing with 95% ethanol. The combined extract was evaporated under reduced pressure at 40°C to give 14 g.

Aqueous Decoction (E7):
100 g of air-dried powdered leaves were exhaustively extracted by refluxing three times with distilled water. The combined extract was evaporated under reduced pressure at 40°C.

Isolation of Flavonoids:
As E5 is the largest weight obtained, 17 g were separated by ascending PPC using Whatmann No. 3 sheets with S₁ as the developing system.
Compounds F₁ and F₂ were obtained from band 1 using Sephadex LH-20 column and S₂ as eluant. F₁ was purified by polyamide column using S₃ as eluant while F₂ was purified by cellulose column and developed with S₅ followed by Sephadex LH-20 column and developed with S₄.
Compound F₃ was obtained from band 2 using Sephadex LH-20 column and S₄ as eluant then polyamide column using S₅ as eluant. Finally F₃ was isolated from preparative TLC silica gel F₂₅₄ with S₆ as a solvent system.

Acid Hydrolysis of Glycosides (Mabry et al., 1970; Harborne, 1973):
Complete Acid Hydrolysis:
5 mg of each compound was dissolved in 5 mL methanol and an equal volume of 2N HCl aqueous solution was added. The mixture was refluxed on a boiling water bath for 3 hours. The methanol was evaporated and the aglycone was extracted with ether. The aqueous layer containing the sugar part was evaporated to dryness and the residue was dissolved in 10% isopropanol solution.

Identification of the Aglycone:
The ether extract of each compound containing the aglycone moiety was applied on paper chromatogram along side with authentic flavonoid aglycones using the solvent systems S₁ and S₅, visualized under UV, exposed to ammonia vapour and sprayed with AlCl₃ (Mabry et al., 1970).

Identification of the Sugars:
The isopropanol solution of each compound containing the sugar moiety was applied on paper chromatogram along side with authentic sugars using the solvent systems S₁ and S₅. After development, they were visualized by spraying with aniline hydrogen phthalate reagent (Partridge, 1949) and heating at 110°C for 10 min (Harborne, 1973).
**Determination of Median Lethal Dose (LD$_{50}$):**

The median lethal dose (LD$_{50}$) of all extracts of *C. insignis* HBK. was determined according to the method described by Karber (1931). Male albino mice were divided into groups, each of six animals. Preliminary experiments were undertaken to determine the minimal dose that kills all animals (LD$_{100}$) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in-between these two doses, and each dose was injected into a group of six animals by subcutaneous injection. The mice were observed for 24 hours, symptoms of toxicity and mortality rates in each group were recorded, and LD$_{50}$ was calculated for each extract.

**Determination of Acute Anti-inflammatory Activity:**

Paw swelling, or footpad oedema, is a convenient method for assessing acute anti-inflammatory activity (Winter et al., 1962). This model uses carrageenan as an irritant to induce paw oedema. Typically, test materials are assessed for acute anti-inflammatory activity by examining their ability to reduce or prevent the development of carrageenan-induced paw swelling. Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, reduce paw swelling in a dose-dependent manner.

Fifty four adult male albino rats were divided into nine groups, each of six animals, as follows:
1. First group: Rats that received 1 ml saline serving as control.
2. Second, third, forth, fifth, sixth, seventh and eighth groups: Rats that received 100 mg/kg b.wt. of E1 – E7, respectively.
3. Ninth group: Rats that received 20 mg/kg b.wt. of the reference drug (indomethacin).

One hour after oral administration, all animals were given a sub-plantar injection of 0.1 ml of 1% carrageenan solution in saline in the right hind paw and 0.1 ml saline in the left hind paw. Four hours after drug administration, the rats were sacrificed. Both hind paws were, separately, excised and weighed. Calculations:

The percentage oedema was calculated according to the following equation:

\[
\% \text{ Oedema} = \frac{\text{wt. of right paw} - \text{wt. of left paw}}{\text{wt. of left paw}} \times 100
\]

**Determination of Acute Antihyperglycemic Activity:**

Male albino rats of the Sprague Dawely strain were injected intraperitoneally with alloxan (150 mg/kg b.wt.) to induce diabetes mellitus (Eliasson and Samet, 1969). Hyperglycemia was assessed after 72 hours by measuring blood glucose (Trinder, 1969) and after two months.

Animals were divided into nine groups:
1. First group: Diabetic rats that serve as positive control.
2. Second, third, forth, fifth, sixth, seventh and eighth groups: Diabetic rats that received 100 mg/kg b.wt. of E1 – E7, respectively.
3. Ninth group: Diabetic rats that received 150 mg/kg b.wt. of metformin as a reference drug.

At the end of each study period, blood samples were collected from the retro orbital venous plexus through the eye canthus of anesthetized rats after an overnight fast. Serum was isolated by centrifugation and the blood glucose level was measured (Trinder, 1969).

**Determination of in vitro Antioxidant Activity:**

The potential antioxidant activity of E7 was assessed on the basis of the scavenging activity of the stable DPPH free radical using ESR spectroscopy. Reaction mixtures contained 100 μl test samples (0.5 mg/ml) and 100 μl of DPPH ethanolic solution (5´10$^{-4}$ M). Due to its paramagnetic properties, DPPH exhibits an ESR signal. ESR spectra were obtained with ESR spectrometer using micro-sampling pipettes at room temperature (Calliste et al., 2001). All spectra were recorded and the inhibition ratio was calculated using the following equation:

\[
\text{Inhibition Ratio} = \frac{\text{Ref-Extract}}{\text{Ref}} \times 100
\]

Ref = the double integral of the reference signal (DPPH).
Extract = the double integral of the test signal (DPPH + E7).
**Determination of in vivo Antioxidant Activity:**

Sixty adult male albino rats were divided into ten groups, each of six animals, as follows:

1. First group: received 1 ml saline and kept as negative control.

2. Second group: Diabetic rats that kept untreated.

3. Third, forth, fifth, sixth, seventh, eighth, and ninth groups: Diabetic rats that received 100 mg/kg b.wt. of E1 – E7, respectively for seven days.

4. Tenth group: Diabetic rats that received 7.5 mg/kg of vitamin E as a reference drug (positive control).

At the end of the experiment, blood glutathione was estimated using biodiagnostic kits (Beutler et al., 1963).

**Determination of Hepatoprotective Activity:**

**Induction of Liver Damage:**

Liver damage in rats was induced according to the method of Klassen and Plaa (1969) by intraperitoneal injection of 5 ml/kg of 25% CCl4 in liquid paraffin.

**Experimental Design:**

Ninety male albino rats were randomly divided into nine groups each of ten rats.

Animal groups:

1. First group: control group received a daily oral dose of 1 ml saline for one month before and after liver damage (negative control).

2. Second, third, forth, fifth, sixth, seventh and eighth groups: liver damaged rats pretreated with daily oral dose of 100 mg/kg b.wt. of E1 – E7, respectively for one month. Administration of the extracts was continued after liver damage for another month.

3. Ninth group: liver damaged rats pretreated with daily oral dose of 25 mg/kg b.wt. silymarin as a standard. Administration of the drug was continued after liver damage for another month.

Followed by overnight fast, whole blood was obtained from the retro orbital venous plexus through the eye canthus of anesthetized rat. Blood samples were collected at zero time and one month before CCl4 injection and 72 hours and one month after CCl4 injection. Serum was isolated by centrifugation. Serum AST, ALT (Thewfweld, 1974), and ALP (Kind and King, 1954) were measured.

**Statistical Analysis:**

Results are expressed as mean ± S.E. The data was statistically analyzed using the Student’s “t” test (Snedecor and Cochran, 1971). Results with p< 0.01 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Yield of Successive Extracts:**

Table 1 represents the yield of E1 – E5 of *C. insignis* leaves. E1 gave the highest yield followed by E5 then E2, E3 and E4.

<table>
<thead>
<tr>
<th>Extract / Fraction</th>
<th>Wt. (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>38.00</td>
</tr>
<tr>
<td>E2</td>
<td>8.83</td>
</tr>
<tr>
<td>E3</td>
<td>6.61</td>
</tr>
<tr>
<td>E4</td>
<td>4.03</td>
</tr>
<tr>
<td>E5</td>
<td>17.25</td>
</tr>
</tbody>
</table>

E1= Petroleum ether extract, E2= Ether fraction, E3= Chloroform fraction, E4= Ethyl acetate fraction, E5= n-butanol fraction.

**Isolated Flavonoids:**

Compound F1 isolated as yellow amorphous powder (15 mg), Rf= 0.41 and 0.40 in solvent systems S1 and S2, respectively. Under UV light, it appeared as a dark purple spot changed to yellow on exposure to ammonia vapor or spraying with AlCl3.

UV \( \lambda_{max} \) (nm): MeOH (257, 268sh, 299sh, 356), NaOMe (273, 325, 411), AlCl3 (273, 302sh, 421), AlCl3/HCl (265, 300, 361sh, 400), NaOAc (273, 325, 401), NaOAc/H3BO3 (263, 300, 380).
UV spectra shows a bathochromic shift in band I (55 nm), on addition of NaOMe, accompanied with an increase in intensity which was an evidence for free 4'-OH. Similarly, the bathochromic shift in band II (16 nm), on addition of NaOAc, referred to a free 7-OH. A bathochromic shift of 65 nm in band I in the presence of AlCl₃ relative to band I in MeOH was observed indicating that 3-position is substituted. In addition, a hypsochromic shift of more than 20 nm of band I in AlCl₃/HCl spectrum relative to band I in the spectrum of AlCl₃ confirmed the presence of ortho-dihydroxy groups in B ring (Markham, 1982).

\[ \delta (\text{ppm}) = 7.51 (m, 2H, H-2', 6'), 6.15 (d, J = 2.5 Hz, 1H, H-8), 6.15 (d, J = 2.5 Hz, 1H, H-6), 5.28 (d, J = 6.6 Hz, 1H, H-1''), 4.39 (s, 1H, H-1'''), 3 - 3.9 (sugar protons), 1.19 (d, J = 6.0 Hz, 3H, H-6'''). \]

The \(^1\)H-NMR spectrum revealed the presence of glucose, rhamnose and quercetin. The spectrum showed an overlapping signal at \( \delta 7.51 \) for H-2', 6' and a doublet at \( \delta 6.81 (J = 8.4 \text{ Hz}) \) for H-5' due to ortho-coupling with H-6' and two aromatic protons at \( \delta 6.34 \) and 6.15, each proton assigned for H-8 and H-6, respectively. A \( \beta \)-D-rutinoside moiety at C-3 was deduced from the two anomic proton signals at \( \delta 5.28 (d, J = 6.6 \text{ Hz}) \) and \( \delta 4.39 \) (singlet) together with a doublet of three protons at \( \delta 1.19 (J = 6.0 \text{ Hz}) \) for Me-6'''. A \( 1''' - 6''' \) interglycosidic linkage was followed from the characteristic upfield location of H-1''' as a broad singlet at \( \delta 4.39 \).

The structure was confirmed by determination of ESI-MS (negative mode) \( m/z: 609 \) [M'-H] (M.wt. of quercetin rutinoside-H) and \( m/z 301 \) [M'-H-glucose-rhamnose] (M.wt. of quercetin-H).

Complete acid hydrolysis yielded glucose and rhamnose in the aqueous phase and quercetin in the organic phase (CoPC in S₁, S₂ and S₃ using specific spray reagents).

Therefore compound F1 was identified as Rutin [quercetin-3-O-\( \alpha \)-L-rhamnopyranosyl-(1''''-6'')-\( \beta \)-D-glucopyranoside]. This is the first report for its isolation from the genus Chorisia.

Compound F2 isolated as yellow amorphous powder (25 mg), \( R_f = 0.49 \) and 0.46 in solvent systems S₁ and S₃, respectively. Under UV light, it appeared as a dark purple spot changed to yellow on exposure to ammonia vapor or spraying with AlCl₃.

UV \( \lambda_{\text{max}} \) (nm): MeOH (256, 268sh, 349), NaOMe (264, 299, 401), AlCl₃ (274, 297sh, 332, 426), AlCl₃/HCl (269, 296, 357, 389), NaOAc (259, 267sh, 360, 403), NaOAc/H₂BO₃ (260, 373).

A bathochromic shift in band I (52 nm), on addition of NaOMe, accompanied with an increase in intensity was an evidence for free 4'-OH. No bathochromic shift was observed in the presence of NaOAc; indicating a substitution at 7-OH. In addition, a hypsochromic shift of more than 20 nm of band I in AlCl₃/HCl spectrum relative to band I in the spectrum of AlCl₃ confirmed the presence of ortho-dihydroxy groups in B ring (Markham, 1982).

\[ \delta (\text{ppm}) = 7.38 (d, J = 8.55, 2.5 Hz, 2H, H-2', 6'), 6.83 (s, 1H, H-8), 6.70 (s, 1H, H-3), 6.32 (s, 1H, H-6), 5.19 (d, J = 6.6 Hz, 1H, H-1''), 5.07 (s, 1H, H-1'''), 3.1 - 4.0 (sugar protons), 1.18 (d, J = 6.0 Hz, 3H, H-6'''). \]
The 1H-NMR spectrum revealed the presence of glucose, rhamnose and luteolin. The spectrum showed two doublets at $\delta$ 7.38 with ($J$ = 8.55, 2.5 Hz) for H-2', 6' and a doublet at $\delta$ 6.85 ($J$ = 8.6 Hz) for H-5 due to ortho-coupling with H-6'. Also a singlet at $\delta$ 6.70 corresponding to H-3, two protons broad singlet at $\delta$ 6.83 and 6.32, assigned to H-8 and H-6, respectively.

A $\beta$-D-rutinoside moiety at C-7 was deduced from the two anomic proton signals at $\delta$ 5.19 (d, $J$ = 6.6 Hz) and $\delta$ 5.07 (singlet) together with a doublet of three protons at $\delta$ 1.18 ($J$ = 6.0 Hz) for Me-6'. 

A 1`''-6''-interglycosidic linkage was followed from the characteristic upfield location of H-1``'' as a broad singlet at $\delta$ 5.07.

The structure was confirmed by determination of ESI-MS (negative mode): m/z 593 [M'-H] (M.wt. of luteolin rutinoside-H), m/z 447 [M'-H-rhamnose] (M.wt. of luteolin glucoside-H) and m/z 285 [M'-H-rhamnose-glucose] (M.wt. of luteolin-H).

Complete acid hydrolysis yielded glucose and rhamnose in the aqueous phase and luteolin in the organic phase (CoPC in $S_1$, $S_2$, and $S_3$ using specific spray reagents).

Therefore compound F2 was identified as Luteolin-7-O- $\beta$-D-rutinoside [luteolin-7-O-\alpha-L-rhamnopyranosyl-(1''''-6'')]-O-$\beta$-D-glucopyranoside]. This is the first report for its isolation from the genus Chorisia.

Compound F3 isolated as pale yellow amorphous powder (20 mg), $R_f$ = 0.59 and 0.49 in solvent systems $S_1$ and $S_3$, respectively. Under UV light, it appeared as a dark purple spot changed to yellow on exposure to ammonia vapor or spraying with AlCl$_3$.

UV $\lambda_{max}$ (nm): MeOH (268, 335), NaOMe (255sh, 268, 301sh, 389), AlCl$_3$ (276, 299, 345, 380), AlCl$_3$/HCl (276, 299, 345, 380), NaOAc (257sh, 267, 340, 398), NaOAc/H$_3$BO$_3$ (267, 339).

The UV spectral data in MeOH, after addition of NaOMe, showed a bathochromic shift of 54 nm in band I with increased intensity; confirming the presence of a free 4'-OH. No bathochromic shift was observed in the presence of NaOAc; indicating a substitution at 7-OH. Absence of ortho-dihydroxy groups in either ring A or B was assured by the absence of bathochromic shift in band I with NaOAc/H$_3$BO$_3$ and in band I in AlCl$_3$/HCl (Markham, 1982).

$^1$H-NMR (500 MHz, DMSO-d$_6$): $\delta$ (ppm) = 7.90 (d, $J$ = 8.6 Hz, 2H, H-2', 6'), 6.92 (d, $J$ = 8.6 Hz, 2H, H-3', 5'), 6.82 (s, 1H, H-3), 6.76 (d, $J$ = 2.5 Hz, 1H, H-8), 6.33 (d, $J$ = 2.5 Hz, 1H, H-6), 5.19 (d, $J$ = 7.45 Hz, 1H, H-1''), 5.09 (s, 1H, H-1'''), 3.12 - 4.09 (sugar protons), 1.19 (d, $J$ = 6.0 Hz, 3H, H-6''').

The 1H-NMR spectrum revealed the presence of glucose, rhamnose and apigenin. The spectrum showed four aromatic protons appearing as two doublets at $\delta$ 7.90 with ($J$ = 8.6 Hz) and $\delta$ 6.92 with ($J$ = 8.6 Hz) due to ortho-coupled protons, were assigned to H-2', 6' and H-3', 5', respectively. Also a singlet at $\delta$ 6.82 corresponding to H-3, two protons broad singlet at $\delta$ 6.76 and 6.33, assigned to H-8 and H-6, respectively. A $\beta$-D-rutinoside moiety at C-7 was deduced from the two anomic proton signals at $\delta$ 5.19 (d, $J$ = 7.45 Hz) and $\delta$ 5.09 (singlet) together with a doublet of three protons at $\delta$ 1.19 ($J$ = 6.0 Hz) for Me-6''. A 1''''-6''-interglycosidic linkage was followed from the characteristic upfield location of H-1'''' as a broad singlet at $\delta$ 5.09.

$^{13}$C-NMR (500 MHz, DMSO-d$_6$): $\delta$ (ppm) = 182.5 (C-4), 165.3 (C-7), 164.8 (C-2), 162.3 (C-4'), 161.5 (C-5), 157.4 (C-9), 129.1 (C-2', 6'), 121.2 (C-1''), 116.6 (C-3', 5'), 105.9 (C-10), 103.9 (C-3'), 101.0 (C-1'''), 99.6 (C-6), 98.6 (C-1'''''), 95.0 (C-8), 77.6 (C-3''), 77.5 (C-5''), 72.4 (C-2'', 4'''), 72.3 (C-3'''), 71.8 (C-2'''), 70.9 (C-4'''), 68.7 (C-5'''), 61.0 (C-6''), 18.6 (C-6''). It revealed the presence of apigenin, glucose and rhamnose. C-7 upfield shifted at $\delta$ 165.3 due to glycosylation.

The structure was confirmed by determination of ESI-MS (negative mode) m/z: 577 [M'-H] (M.wt. of apigenin rutinoside-H) and m/z 269 [M'-H-glucose-rhamnose] (M.wt. of apigenin-H).

Complete acid hydrolysis yielded glucose and rhamnose in the aqueous phase and apigenin in the organic phase (CoPC in $S_1$, $S_2$, and $S_3$ using specific spray reagents).

Therefore compound F3 was identified as Apigenin-7-O- $\beta$-D-rutinoside [apigenin-7-O-\alpha-L-rhamnopyranosyl-(1''''-6'')]-O-$\beta$-D-glucopyranoside]. This is the first report for its isolation from the genus Chorisia.

**Determination of Median Lethal Dose (LD$_{50}$):**

Table 2 represents LD$_{50}$ of all extracts. All the extracts were safe showing LD$_{50}$ more than 5 g/kg b.wt. E6 was the safest one followed by E7 then E4, E2, E5, E1 and finally E3. The results indicate the low toxicity of the plant.

**Determination of Acute Anti-inflammatory Activity:**

Table 3 illustrates a significant inhibition of the rat paw oedema induced by carrageenan due to the anti-inflammatory effect exhibited by all extracts. The results indicate that E6 has the highest activity followed by E7 and E4, giving relative potencies more than 85%, in comparison with indomethacin then E2, E5 and E3 which showed relative potencies more than 50%. The least oedema reduction was exhibited by E1 which showed 46% relative potency.
Table 2: LD₅₀ of different extracts and successive fractions of the 70% ethanol extract of Chorisia insignis leaves.

<table>
<thead>
<tr>
<th>Extract / Fraction</th>
<th>LD₅₀ g/kg b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>6.3</td>
</tr>
<tr>
<td>E2</td>
<td>6.7</td>
</tr>
<tr>
<td>E3</td>
<td>5.4</td>
</tr>
<tr>
<td>E4</td>
<td>7.1</td>
</tr>
<tr>
<td>E5</td>
<td>6.5</td>
</tr>
<tr>
<td>E6</td>
<td>7.8</td>
</tr>
<tr>
<td>E7</td>
<td>7.5</td>
</tr>
</tbody>
</table>

E1= Petroleum ether extract, E2= Ether fraction, E3= Chloroform fraction, E4= Ethyl acetate fraction, E5= n-butanol fraction, E6= Total extract, E7= Aqueous extract.

The significant anti-inflammatory activity of these extracts could possibly be attributed to their flavonoidal content.

Several mechanisms of action have been proposed for the bioflavonoid actions in reducing inflammation. Apigenin inhibits the collagenase activity involved in rheumatoid arthritis (RA) and suppresses lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RA. Pretreatment with apigenin also attenuates LPS-induced cyclooxygenase-2 (COX-2) expression which is elevated in the synovial lining layer, the subsynovial synoviocytes, the vascular endothelial cells and mononuclear inflammatory cells in patients with RA or osteoarthritis (OA) (Lee et al., 2007). In addition, apigenin profoundly reduces the tumor necrosis factor-α (TNF-α)-induced adhesion of monocytes to human umbilical vein endothelial cell (HUVEC) monolayer which is elevated in early RA (Lee et al., 2007).

It has been suggested that intradermal application of apigenin-7-glucoside inhibited skin inflammation caused by xanthine-oxidase and cumene hydroperoxide (Fuchs and Milbradt, 1993).

Luteolin may be a potent selective inhibitor of COX-2 and the inhibition is attributable to its down-regulation of the mRNA expression of COX-2 in inflammatory responses (Ziyan et al., 2007). It inhibits arachidonic acid- and 12-O-tetradecanoylphorbol-13-acetate-induced ear edema (Ueda et al., 2002). Luteolin and its derived glycosides have inhibitory activity against enzymes for the synthesis of thromboxane and leukotriene involved in inflammation (Odontuya et al., 2005).

Table 3: Effect of different extracts and successive fractions of the 70% ethanol extract of Chorisia insignis leaves on carrageenan-induced rat hind paw oedema.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose in mg/kg b.wt.</th>
<th>% Oedema</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.E.</td>
<td>% of change</td>
</tr>
<tr>
<td>Control</td>
<td>1 ml saline</td>
<td>61.3 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td>E1</td>
<td>100</td>
<td>43.2 ± 1.9 *</td>
<td>29.5</td>
</tr>
<tr>
<td>E2</td>
<td>100</td>
<td>32.3 ± 1.2 *</td>
<td>47.3</td>
</tr>
<tr>
<td>E3</td>
<td>100</td>
<td>39.6 ± 1.3 *</td>
<td>35.4</td>
</tr>
<tr>
<td>E4</td>
<td>100</td>
<td>27.8 ± 0.9 *</td>
<td>54.7</td>
</tr>
<tr>
<td>E5</td>
<td>100</td>
<td>35.4 ± 1.6 *</td>
<td>42.2</td>
</tr>
<tr>
<td>E6</td>
<td>100</td>
<td>26.1 ± 0.7 *</td>
<td>57.4</td>
</tr>
<tr>
<td>E7</td>
<td>100</td>
<td>26.9 ± 0.8 *</td>
<td>56.1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>20</td>
<td>22.1 ± 0.5 *</td>
<td>64.0</td>
</tr>
</tbody>
</table>

E1= Petroleum ether extract, E2= Ether fraction, E3= Chloroform fraction, E4= Ethyl acetate fraction, E5= n-butanol fraction, E6= Total extract, E7= Aqueous extract.

* Significantly different from the control group at p< 0.01

% of change calculated as regards the control group.

Potency calculated relative to indomethacin.

Quercetin inhibits macrophage proliferation and activation and cytokine expression induced by LPS that are extensively involved in the initiation and progression of the inflammation process (Camalada et al., 2005). Intraportal administration of rutin and quercetin inhibit both acute and chronic phases of inflammation (Selloum et al., 2003; Guardia et al., 2001).

**Determination of Acute Antihyperglycemic Activity:**

Results are cited in Figs. 2 and 3. The results revealed that the highest activity was exhibited by E6 giving 80% potency relative to metformin followed by E7 and E4 giving more than 68% relative potency which could possibly be attributed to their flavonoidal content and phenolic compounds. E5 and E3 have moderate activity (more than 50% relative potency), while E1 and E2 have the least activity (less than 50% relative potency). It was reported that apigenin-6-C-(2”-O-α-l-rhamnopyranosyl)-β-l-fucopyranoside has an acute effect on blood
glucose lowering in diabetic rats and stimulates glucose-induced insulin secretion after oral treatment in hyperglycemic rats (Cazarolli et al., 2008). Apigenin-6-C-glucosyl-7-O-glucoside has a mixed competitive inhibition on activities of α-glucosidase and sucrase (Subhabrata et al., 2009).

Also luteolin and luteolin-7-O-glucoside have inhibitory activities against α-glucosidase and α-amylase. Luteolin is stronger than acarbose, the most widely prescribed drug, in inhibitory potency, suggesting that it has the possibility to effectively suppress postprandial hyperglycemia in patients with non-insulin dependent diabetes mellitus (Kim et al., 2000). Luteolin-5-rutinoside increases pancreatic insulin in streptozotocin (STZ)-induced diabetic rats. When both luteolin-5-rutinoside and glibenclamide, an anti-diabetic drug, were administered concurrently to STZ-diabetic rats, a marked antidiabetic activity was achieved (Zarzuelo et al., 1996).

Intraperitoneal injection of quercetin in STZ-induced diabetic rats significantly decreased the plasma glucose level and increases insulin release. Hepatic glucokinase (a glucose sensor, triggering shifts in metabolism or cell function in response to rising or falling levels of glucose) activity was significantly increased upon quercetin treatment (Vessal et al., 2003).

Also oral administration of rutin to STZ-induced diabetic rats significantly decreased fasting plasma glucose and increased insulin level (Kamalakkannan and Prince, 2006).

**Fig. 2:** Acute effect of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of Chorisia insignis leaves on blood glucose level in rats after 8 weeks as compared with metformin (150 mg/kg b.wt.).

**Fig. 3:** Potency of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of Chorisia insignis leaves on blood glucose level in rats after 8 weeks relative to metformin (150 mg/kg b.wt.).
Determination of in vitro Antioxidant Activity:

Table 4 and Fig. 4 illustrate the double integration areas of DPPH by ESR calculated after the addition of the inhibitor (extract). E6 had a significant activity as free radical scavenger; it showed 44% inhibition in comparison with vitamin C (100%).

Determination of in vivo Antioxidant Activity:

Table 5 revealed that E4, E5, E6 and E7 showed significant antioxidant activities comparable to vitamin E. E6 had the best activity (2.6% of change from control) followed by E4 (3.3% of change from control) and E7 (3.6% of change from control) while E5 had the least activity (6.3% of change from control). The relative potencies of E6, E4, E7 and E5 were 98.6%, 98.1%, 97.8% and 95.0%, respectively as compared with vitamin E.

Table 4: In vitro antioxidant activity of the total extract of *Chorisia insignis* leaves.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration</th>
<th>Double Integration Area (DIA)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>5′10⁻⁴</td>
<td>4474259.6</td>
<td>0</td>
</tr>
<tr>
<td>E6</td>
<td>5′10⁻⁴</td>
<td>2490558</td>
<td>44</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5′10⁻⁴</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

DPPH= 1,1-diphenyl-2-picrylhydrazil, E6= Total extract.

Table 5: Antioxidant effect of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of *Chorisia insignis* leaves as compared with Vitamin E (7.5 mg/kg b.wt.).

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glutathione (mg %)</th>
<th>% of change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.4 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>Diabetic</td>
<td>21.6 ± 0.4 *</td>
<td>40.7</td>
</tr>
<tr>
<td>Diabetic + E1</td>
<td>29.7 ± 0.8 *</td>
<td>18.4</td>
</tr>
<tr>
<td>Diabetic + E2</td>
<td>31.2 ± 1.5 *</td>
<td>14.3</td>
</tr>
<tr>
<td>Diabetic + E3</td>
<td>30.1 ± 1.2 *</td>
<td>17.3</td>
</tr>
<tr>
<td>Diabetic + E4</td>
<td>35.2 ± 0.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Diabetic + E5</td>
<td>34.1 ± 0.7</td>
<td>6.3</td>
</tr>
<tr>
<td>Diabetic + E6</td>
<td>35.4 ± 0.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Diabetic + E7</td>
<td>35.1 ± 0.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Diabetic + Vitamin E</td>
<td>35.9 ± 1.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

E1= Petroleum ether extract, E2= Ether fraction, E3= Chloroform fraction, E4= Ethyl acetate fraction, E5= n-butanol fraction, E6= Total extract, E7= Aqueous extract.

* Statistically significant different from control at P< 0.01

Potency calculated relative to vitamin E.

Glutathione (GSH) is the body’s most abundant natural antioxidant. A lack of GSH has been shown to leave the body more vulnerable to damage by free radicals, thus speeding up oxidation of the body. The diabetogenic process appears to be caused by immune destruction of the beta cells; part of this process is apparently mediated by production of active oxygen species. Diabetes can be produced in animals by the drugs such as alloxan which result in the production of active oxygen species (Oberley, 1988). A simultaneous fall in blood GSH was observed following the injection of diabetogenic doses of alloxan into rabbits (Leech and Bailey, 1945). Therefore, blood glutathione was estimated in alloxan-induced diabetic rats for studying the antioxidant activity.

The antioxidant capabilities of 4 ,5,7-Trihydroxyflavone’s lie in the H⁺ donating potential of its aromatic OH-group. It has been shown to reverse the oxygen radical-generated DNA damage from hydrogen peroxide, hydroxyl radicals, superoxide radicals or singlet oxygen (Morrissey *et al.*, 2005). It also acts as co-antioxidants by facilitating antioxidant activity of other compounds in the body (Rezzani, 2006).

Apigenin is known to possess antioxidant activities with a strong radical scavenging capacity (Soares and Azevedo, 2006). Apigenin reduces cyclosporine-A (CsA) induced changes in total antioxidants in rats (Nagaraja *et al.*, 2009). It is known that CsA causes oxidative damage in the organ systems in the body due to the generation of reactive oxygen species (ROS) (Tirkey *et al.*, 2005). Treatment with apigenin along with CsA increased the total antioxidants in serum. Treatment of rats with apigenin alone showed a significant improvement in the total antioxidant levels compared to control rats. Improvement of glutathione peroxidase enzyme level in apigenin treated group further demonstrates the antioxidant effect of apigenin (Soares and Azevedo, 2006).

Luteolin and its related glycosides are reactive against hydrogen peroxide radicals. This reactivity is dependent on the presence of ortho-dihydroxy groups at the B ring and OH substitution pattern at C-5 position of the A ring (Odontuya *et al.*, 2005).
Luteolin possesses a high DNA protective capacity against free radicals generated by \( H_2O_2 \) (Romanová et al., 2001). Pretreatment with quercetin increases the level and the activity of GSH in mice (Molina et al., 2003). Quercetin has the ability to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical (Boots et al., 2008). Rutin significantly increases the antioxidant status in mouse liver (Gao et al., 2002).

**Determination of Hepatoprotective Activity:**

It was revealed that pretreatment of rats with E6, E7 or E4 for one month before and after liver damage, induced by intraperitoneal injection of \( CCl_4 \), resulted in significant decrease in AST, ALT and ALP as compared with silymarin (Figs. 5, 6 and 7).

**Fig. 4:** ESR spectrum of DPPH and the total extract of Chorisia insignis leaves.

E6 showed the highest percent protection (91.33% and 91.41% AST, 81.23% and 93% ALT and 90.16% and 88.02% ALP after 72 hours and 30 days respectively) followed by E7 (84.23% and 88.03% AST, 77.3% and 89.4% ALT and 86.74% and 85.63% ALP after 72 hours and 30 days respectively) and E4 (87.44% and 90.04% AST, 73.07% and 89.31% ALT and 73.49% and 84.13% ALP after 72 hours and 30 days, respectively) which could possibly be attributed to their flavonoidal content and phenolic compounds such as apigenin, luteolin and quercetin and their derived glycosides.

The results show that \( CCl_4 \) administration causes severe acute liver damage in mice, demonstrated by remarkable elevation of serum AST and ALT levels. The increased serum levels of AST and ALT have been attributed to the damaged structural integrity of the liver. This is because they are intracellular enzymes, released into circulation after hepatocyte damage or necrosis (Sallie et al., 1991).

Pretreatment with apigenin was able to suppress the elevation of AST and ALT in a dose-dependent manner *in vivo* and reduce the damage of hepatocytes *in vitro* caused by \( CCl_4 \) in blood serum. The hepatoprotective activity of apigenin is possibly due to its antioxidant properties, acting as scavengers of ROS (Zheng et al., 2005).

\( CCl_4 \)-induced hepatotoxicity was reduced in a dose- and time-dependent manner, as determined by decreased serum aminotransferase activities, upon pretreatment with luteolin in mice (Domitrović et al., 2008). Quercetin has a hepatoprotective effect upon chronic administration in rats with \( CCl_4 \)-induced fibrosis (Pavanato et al., 2003).

Rutin is able to prevent the \( CCl_4 \)-induced rise in serum enzymes confirming its hepatoprotectivity (Janbaz et al., 2002).

Pretreatment with rutin ameliorates the effects induced by radiation including serum liver enzymes (ALP, AST and ALT) activities (Radwan et al., 2008).
Fig. 5: Effect of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of Chorisia insignis leaves on AST level in liver damaged rats as compared with silymarin (25 mg/kg).

Fig. 6: Effect of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of Chorisia insignis leaves on ALT level in liver damaged rats as compared with silymarin (25 mg/kg).

Fig. 7: Effect of different extracts and successive fractions of the 70% ethanol extract (100 mg/kg b.wt.) of Chorisia insignis leaves on ALP level in liver damaged rats as compared with silymarin (25 mg/kg).

Conclusion:
In the present study, the n-butanol fraction of the 70% ethanol extract of the air-dried powdered leaves of *C. insignis* HBK. was purified by PC then CC and/or PTLC to afford three flavonoids (compounds F1, F2 and F3). These compounds were identified as Rutin (F1), Luteolin-7-O-β-D-rutinoside (F2) and Apigenin-7-
O-β-D-rutinoside (F3). This is the first report for the isolation of the three compounds from the genus *Chorisia*. The LD₅₀ of the total extract, aqueous extract and the successive fractions of the 70% ethanol extract revealed the low toxicity of the plant.

Oral administration of *C. insignis* leaf extracts to mice inhibits inflammation as indicated by inhibition of the rat paw oedema induced by carrageenan. These results suggested that the total extract, aqueous extract and the ethyl acetate fraction may be useful for therapeutic management of inflammatory diseases.

It was also found that the total extract, aqueous extract and the ethyl acetate fraction decreased blood glucose level in alloxan-induced diabetic male albino rats as compared with metformin.

In addition, a significant antioxidant effect was exhibited by the total extract, giving the highest activity, followed by the ethyl acetate fraction and the aqueous extract then n-butanol fraction, in comparison with vitamin E. The reactivity of these extracts was evaluated by the increase in blood glutathione, the body’s natural antioxidant, in alloxan-induced diabetic rats.

Moreover, treatment with the total extract, aqueous extract or the ethyl acetate fraction significantly decreased the CCl₄-induced elevation in biochemical parameters (AST, ALT and ALP). These findings suggested that these extracts were effective in bringing about functional improvement of hepatocytes which could be a result to their antioxidant activities. The study demonstrates that the leaves of *C. insignis* contain flavanoids and phenolic compounds which may have a potential therapeutic approach to hepatoprotective properties.

In conclusion, our results demonstrate the occurrence of anti-inflammatory, antihyperglycemic, antioxidative and hepatoprotective principles in the leaves of *chorisia insignis*.

REFERENCES


