ANNEXURE

Publications

Papers published


Paper Communicated


Paper presented

Screening of antioxidant activity, total phenolics and gas chromatography-mass spectrophotometer (GC-MS) study of ethanolic extract of *Aporosa lindleyana* Baill

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The present study was carried out for the identification of phytochemicals present in the roots of *Aporosa lindleyana* and also to evaluate the total phenol, total flavonoids and antioxidant activity. Total phenol was carried out by Folin Ciocalteu method and the phenolic content was 31.20 mg/100 g of gallic acid equivalent (GE) and the flavonoid content was 203.10±0.9. Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and the roots of *A. lindleyana* showed 19.91 mg/100 g of ascorbic acid equivalent antioxidant capacity (AEAC). The gas chromatography-mass spectrophotometer (GC-MS) study was also carried out and it showed the presence of phytochemicals like 1,2-benzenedicarboxylic acid, diphenyl ester (RT: 11.97), pthalic acid, bis (7 methylcyctyl) ester (RT: 20.86) and squalene (RT: 24.70).

Key words: *Aporosa lindleyana*, antioxidant, flavonoids, gas chromatography-mass spectrophotometer (GC-MS), 2,2-diphenyl-1-picrylhydrazyl (DPPH).

INTRODUCTION

*Aporosa lindleyana* belongs to the family of Euphorbiaceae, is a much branched, evergreen, glabrous tree, grown in India and Sri Lanka. It possesses antioxidant activity (Badami et al., 2005) and hepatoprotective effect (Ramakrishnan et al., 2010) and also having antihyperglycemic effect (Jayakar and Suresh, 2003). So far, very few research works were carried out in this plant. *A. lindleyana* Baill have much medicinal properties such as diuretic, antiviral and a good analgesic. Its leaves are used to treat diabetics. Its bark and roots were used to treat headache, fever and jaundice. Decoctions of roots are used to treat insanity, seminal loss and excessive thirst.

The preliminary phytochemical studies reveal the presence of phytosterol, alkaloids and flavonoids.

Polyphenolic compounds have high antioxidant potential, the antioxidant activity of *A. lindleyana* was investigated by employing various in vitro tests. The present study was carried out to investigate the antioxidant activity, flavonoids and total phenolic content of *A. lindleyana*. In addition, chemical constituents of *A. lindleyana* was analyzed by gas chromatography-mass spectrophotometer (GC-MS).

MATERIALS AND METHODS

Collection and processing of plant material

The roots of *A. lindleyana* was collected from Keeriparai, Kanyakumari District, Tamilnadu during the month of January 2009. The specimen was identified by Dr. V. Chelladurai, Taxonomist, Department of Ayurvedic Sciences, Tirunelveli District. The roots were cleansed and shade dried for a weak and grounded into uniform powder. 1 g of plant material was added to 20 ml of aqueous ethanol (20% v/v) for 18 h at room temperature. The
Table 1. Total phenols, flavonoids and antioxidant activity in the roots of *A. lindleyana*.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Parameter analysed</th>
<th>Value obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total Phenols (mg/100 g) GE*</td>
<td>31.20±0.2</td>
</tr>
<tr>
<td>2</td>
<td>Total flavonoids (mg/100 g) GE*</td>
<td>203.10±0.9</td>
</tr>
<tr>
<td>3</td>
<td>Antioxidant activity ((mg/100 g) AEAC**)</td>
<td>19.91±0.5</td>
</tr>
</tbody>
</table>

The values are mean value of three replicates, *Gallic acid equivalent, **Ascorbic acid equivalent antioxidant capacity.

Extracts was filtered and used for the estimation of total phenols and antioxidant activity.

**Total phenols**

0.5 ml of freshly prepared sample was taken and diluted with 8 ml of distilled water. 0.5 ml of Folin Ciocalteu reagent (1 N) was added and kept at 40°C for 10 min. 1.0 ml of sodium carbonate (20%) was added and kept in dark for one hour. The color was read at 650 nm using a UV-1500 spectrophotometer (Malick et al., 1998). The same procedure was repeated for all standard gallic acid solution and standard curve obtained. The sample concentration was calculated as gallic acid equivalent (GE).

**Total flavonoids**

0.5 ml of ethanolic extract of sample is diluted with 3.5 ml of distilled water at zero time and 0.3 ml of 5% sodium nitrate was added to the tubes. After 5 min, 0.3 ml of aluminium chloride (10%) was added to all the tubes. At the 6th min, 2 ml of sodium hydroxide (1 M) was added to the mixture. Immediately, the contents of the reaction mixture were diluted with 2.4 ml of distilled water and mixed thoroughly, after which absorbance of the mixture was then determined at 510 nm versus a prepared blank. Gallic acid was used as the standard compound for quantification of total flavonoids as mg/100 g (Zhisen et al., 1999).

**Antioxidant activity**

0.1 ml of the freshly prepared sample was taken in the test tubes. 6.0 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM) was added and the tubes kept in dark for one hour. The color was read at 517 nm. The difference in the optical density (O.D) of DPPH solution and DPPH solution + sample was calculated. The decrease in O.D with sample addition is used for the calculation of antioxidant activity. Ascorbic acid standards were prepared in different concentration and antioxidant activity was determined as ascorbic acid equivalent antioxidant capacity (AEAC) mg/100 g of sample (Koleva et al., 2002).

**GC-MS analysis**

**Preparation of extract**

The ratio of *A. lindleyana* was shade dried and 20 g of the powdered roots was soaked in 95% ethanol for 12 h. The extract was filtered through Whatmann filter paper No. 41 along with 2 g sodium sulphate to remove the sediments and traces of water in the filtrate. Before the filter paper along with sodium sulphate was wetted with 95% ethanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and nonpolar phytocomponents of the plant material used. 2 μl of this solution was employed for GC-MS analysis (Merlin et al., 2009).

**GC analysis**

GC-MS analysis was carried out on a GC clarus 500 perkin Elmer system comprising a AOC-20i autosampler and gas chromatography interfaced to a mass spectrophotometer (GC-MS) instrument employing the following condition. Column Elite – 1 fused silica capillary column (30 x 0.25 mm ID x 1EM df, composed of 100% trimethyl poly siloxane) operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 Ei was employed (split ratio of 1:1) injector temperature (280°C). The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10 C/min to 200°C, then 5 C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectrum was taken at 70 eV; a scan interval of 0.5 s fragments from 40 to 550 Da.

**Identification of components**

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

**RESULTS AND DISCUSSION**

**Total phenol and flavonoid content**

Plant polyphenols, a diverse group of phenolic compounds (flavonols, anthocyanins, phenolic acids, etc.) possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1996).

The flavonoid contents of the extracts were expressed in terms of gallic acid equivalent (Table 1). Total phenolic content of the ethanolic extract of *A. lindleyana* root was
Table 2. Phytochemicals and their activity identified in the ethanolic extract of the roots of A. lindleyana by GC-MS.

<table>
<thead>
<tr>
<th>S/N</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>MW</th>
<th>Peak area (%)</th>
<th>Nature of compound</th>
<th><strong>Activity</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.00</td>
<td>1,3-Dioxolane-2-heptanenitrile, 2-methyl-4-o xo-2-phenyl-</td>
<td>C₂H₆N₂O₂</td>
<td>287</td>
<td>1.16</td>
<td>Aromatic nitrile compound</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>2</td>
<td>11.97</td>
<td>2-Benzene dicarboxylic acid, diethyl ester</td>
<td>C₇H₆O₄</td>
<td>362</td>
<td>3.10</td>
<td>Plasticizer compound</td>
<td>Antimicrobial, antifouling</td>
</tr>
<tr>
<td>3</td>
<td>13.08</td>
<td>1,2-Benzene dicarboxylic acid, butyl cyclohexyl ester</td>
<td>C₈H₁₂O₄</td>
<td>304</td>
<td>1.15</td>
<td>Plasticizer compound</td>
<td>Antimicrobial, antifouling</td>
</tr>
<tr>
<td>4</td>
<td>20.86</td>
<td>Phthalic acid, bis(7-methoxyctyl) ester</td>
<td>C₁₁H₁₀O₄</td>
<td>418</td>
<td>10.46</td>
<td>Plasticizer compound</td>
<td>Antimicrobial, antifouling</td>
</tr>
<tr>
<td>5</td>
<td>21.38</td>
<td>4-Methoxymethoxy-hex-1-ene</td>
<td>C₁₂H₁₂O₂</td>
<td>144</td>
<td>4.79</td>
<td>Alkane compound</td>
<td>No activity reported</td>
</tr>
</tbody>
</table>

6  24.70  Squalene  C₂₆H₄₈O₄  410  32.34  Triterpene  Anticancer, antimicrobial, antioxidant, chemopreventive pesticide, anti-tumor sunscreen

7  28.77  Silane, 1,4-phenylenebis[trimethyl- | C₂₆H₄₈Si₂        | 222 | 16.25         | Aromatic silica compound | No activity reported |

8  32.40  2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl- | C₂₆H₄₈O₂Si₂    | 250 | 30.76         | Ketone compound         | No activity reported |

**Source: Dr. Duke's Phytochemical and ethnomedical databases.**

31.20 mg/100 g of GE. The highest value of phenolic content indicates that the plant has high antioxidant activity.

The antioxidant properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation (Benavente-Garcia, 1997). Depending on their structure, flavonoids are able to scavenge practically all known reactive oxygen species (ROS). Total flavonoid content of ethanolic extract of A. lindleyana roots was 203.10 mg/100 g of GE.

GC-MS study

The GC-MS study of roots of A. lindleyana has shown many phytochemicals which contributes to the medicinal activity of the plant (Table 2 and Figure 1). The major component present in the roots of A. lindleyana was 1,3-Dioxolane - 2 - heptanenitrile a'-methyl-e-oxo-2-phenyl (RT:9.00), 1,2-Benzene dicarboxylic acid, diethyl ester (RT:11.97), 1,2 - Benzene dicarboxylic acid butyl cyclohexyl ester (RT:13.08), 1,2-Benzene dicarboxylic acid butyl cyclohexyl ester (RT:20.86), 4-Methoxymethoxyhex-1-ene, Squalene (RT: 24.70) - (Figure 2), Silane 1.4, phenylene bis(trimethyl), 2,4,6 cycloheptatrien-1-one, 3,5-bis-trimethylsilyl found in the roots of A. lindleyana.

Squalene plays a major role in the protection and enhancement of human skin. Like glutathione (GSH), Squalene is one of the few antioxidants manufactured within the body, both of which detoxify, help balance our protective metabolisms and protect us from other threats. Squalene also has anticancer and blood cholesterol lowering effects (Nakagawa et al., 1985).

In our study, Squalene, an antioxidant found in the ethanolic extract, may give antioxidant properties of the A. lindleyana.

Conclusion

Our present study showed that the root extract was rich in antioxidants, phenolics and flavonoids. The GC-MS study also proved many phytochemicals such as Squalene, Phthalic acid and 1,2 benzene dicarboxylic acid, butyl cyclohexyl ester etc., which contributes to the activities like antioxidant, antimicrobial and antifouling activity.

ACKNOWLEDGEMENT

We are immensely thankful to Indian Institute of Crop Processing Technology, Thanjavur and The Management, The Principal of Sri Paramakalyani College, Alwarkurichi for providing all the facilities and constant encouragement during these period of work.
Figure 1. Chromatogram of A. lindleyana roots by GC-MS.

Figure 2. Mass spectrum of 1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester.

Name: 1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester
Formula: C₁₈H₂₄O₄
MW: 304 CAS#: 84-64-0 NIST#: 75987 ID#: 87207 DB: mainlib
Other DBs: None
Contributor: RADIANT CORP
10 largest peaks:
149 999 | 41 104 | 150 92 | 55 89 | 29 50 |
223 44 | 67 44 | 57 37 | 76 37 | 65 30 |
Synonyms:
1. Phthalic acid, butyl cyclohexyl ester
2. Butyl cyclohexyl phthalate
3. Cyclohexyl butyl phthalate
4. Bastex 508
5. 1-Butyl 2-cyclohexyl phthalate #
Name: Squalene
Formula: C30H50
MW: 410 CAS#: 7683-64-9 NIST#: 227620 ID#: 27655 DB: mainlib
Other DBs: None
Contributor: Japan AIST/NIMC Database- Spectrum MS-NW-8230
10 largest peaks:
69 999 | 81 612 | 41 257 | 136 243 | 137 240 |
95 184 | 121 139 | 123 137 | 68 124 | 149 119 |
Synonyms:
1. 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-
2. Squalene
3. Spinacene
4. Supraene
5. (6E, 10E, 14E, 18E)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene

Figure 3. Mass spectrum of squalene.

REFERENCES
Hepato Protective Effect of Aporosa lindleyena on Rifampicin Induced liver Injury in Male Wistar Rats

Ramakrishnan. S1 and Venkataraman. R1

Abstract

The present study was designed to evaluate the hepatoprotective effect of methanol extract of the root of Aporosa lindleyena in Male wistar rats. Rats were divided into five groups, each consist six rats. Among that Group I as normal and Group V as Silymarin toxic control. Biochemical parameters (SGOT, SGPT, ALP, Total Bilirubin etc.) were analysed after rifampicin induced in the Group II and Group III (with Plant extract treatment) whereas Group IV was treated with root powder of Aporosa lindleyana. The resultant values showed that the methanol extract of Aporosa lindleyana has the hepatoprotective effect on male wistar rats.

Keywords: Aporosa lindleyana, Hepatoprotective effect, SGOT, SGPT, Rats.

Introduction

Tuberculosis is one of the most common infectious disease, especially pulmonary tuberculosis is one of the major cause for the adult deaths. Isoniazid (INH) and Rifampicin (RIF) are the first line drugs used in antituberculosis. Chemotherapy is associated with hepatotoxicity. The rate of hepatotoxicity has been reported to much higher in developing countries like India (8 – 30%) compared to that in advanced countries (2 – 3%) with a similar dose schedule. Oxidative stress as one of the mechanism for INH + RIF induced hepatic injury. Hepatotoxicity can affect hundreds of millions of people worldwide, it is the common non neoplastic cause of death among hepatobiliary and digestive disorders.

Serious side effects of the cost of the modern medicine and improper channel of treatment and competitive efficacy of the natural products made the persons through the world to look for the classical plant drugs for the treatment of hepato toxicity. In view of the biological properties and chemical constituents of plant from aporosa roots, it was decided to study the plant Aporosa lindleyana (Euphorbiaceae) which is mainly used in the folk medicine.

Aporosa lindleyana plant is commonly known as vetti (in tamil). This plant is used as a traditional medicine for many ailments. The present work mainly aimed in evaluating this hepatoprotective effect in male wistar rats.

Materials and Methods

Plant materials:

Fresh root of Aporosa lindleyana plant was collected from Keeriparai, Kanyakumari District, Tamilnadu (India) during the month of January 2009. The specimen herbarium (MU/SD/KT 532) was identified by Dr. V. Chelladurai, taxonomist, Department of Ayurvedic Science, Tirunelveli District.

Root extract of Aporosa lindleyana:

The root of the plant was shade dried and powdered. This was extracted with methanol, ethylacetate and sodium hydroxide, the extract were filtered and solvent evaporated by rotary evaporator at reduced pressure. The obtained extract was used for its hepato protective effect on male wistar rats.

Animals:

Male wistar rats weighing 180-200g/body weight were used in this study. The protocol is approved by the Institute of animal ethical committee. Body weights of these rats were monitored sequentially in control and experimental animals for a period of 28 days.

Animals were divided into five groups as Normal control (G-I), Toxic Control INH + RIF (G-II), Treatment control INH + RIF + Root extract of of Aporosa G-III), Treatment control INH + RIF +...
Dried Powder of aporosa (G-IV). Positive control INH + RIF + Silymarin (G-V), where n was the number of animals used included in this study. For hepatoprotective model 50mg/Kg per day of INH and RIF each was used in this study.

INH + RIF's solution were prepared separately in sterile distilled water. INH + RIF were administered orally for 21st days. Both extract and crude root powder were administered orally at a dose of 200mg/kg for 21st days. Liver transaminases, Total Protein, Gamma Glutamyl-3-carboxy-4-nitroanilide (GGTP) and total bilirubin were estimated on 21st days in both control and experimental animals.

**Treatment Protocol:**
- **Group I** = Normal Control (received 1ml of 1% Carboxy Methyl Cellulose (CMC))
- **Group II** = Hepatotoxic control (received 50mg/kg INH+RIF for 21st days) orally
- **Group III** = Treatment control (were given INH+RIF+extract of aporosa for 21st days orally as 200mg/kg suspended with 2ml of 1% CMC
- **Group IV** = Treatment control (were given INH+RIF+powder of aporosa for 21 days orally as 200mg/kg suspended with 2ml of 1% CMC
- **Group V** = Positive control (Standard) were given (NH + RIF + Silymarin 70mg/kg orally for 21st days)

**Methods**
Rats were treated as per protocol. The protocol is approved by the IAC. Body weights of these rats were monitored sequentially in control and experimental animals for a period of 21st days and they were sacrificed after administration of drug on day 21. The blood was collected by retro orbital artery bleeding. Blood samples were kept for 30 minutes without any disturbances in clot activator samples tubes. Then blood samples were centrifuged for 10 minutes at 3000 rpm to separate the serum.

**Biochemical markers**
Aspartate Transaminase (SGOT/AST), Alanine Transaminase (ALT/SGPT), Alkaline Phosphatase (ALP), Total bilirubin, Total Protein and Gamma Glutamyl Trans Peptidase (GGTP) levels were estimated from the serum samples using autoanalyzer results of biochemical analysis are given in Table I.

**Results and Discussion**
There is no previous report about the hepatoprotective activity of this plant. The present investigation reports hepatoprotective effect of methanol extract and powder of this plant.

In the present study hepatotoxic model in wistar rats was successfully produced by administering INH and RIF (50mg/day) orally. During the metabolism of INH, hydrazine is produced directly (from INH) or indirectly (from acetyl hydrazine) from earlier study, it is evident that hydrazine plays a role in INH induced liver damage in rats, which is consistent with the report by Sarichef.a.

The combination of INH and RIF was reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation and cytochrome P450 was thought to be involved in the synergistic effect of Rif on INH. However, It's in INH induced hepatotoxicity is unclarified, as INH itself is an inducer of its CYP2E1.

A small retrospective analysis of patients who developed hepatic dysfunction whilst on antituberculosis drugs, hospitalized in a unit between 1st Jan 1991 31st Dec 1992, was recently undertaken. Out of 1,118 patients who received Rif and INH with or without pyrazinamide and other drugs, 142 developed clinically symptomatic dysfunction. An assumption that the vast majority of hepatic dysfunction episodes should have occurred within two months of commencement of antituberculosis chemotherapy was made, as generally reported. The previous report also says that there did not seem to be clear evidence that INH proves much more injuries than Rif and in this connection, they consider that it is the combination of these two drugs that confer the additive or even synergistic, potential of liver toxicity than either agent alone as conjectured.

**Tables**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total bilirubin</th>
<th>Total protein</th>
<th>ALP</th>
<th>AST</th>
<th>ALT</th>
<th>GGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.62±0.05</td>
<td>0.50±0.08</td>
<td>120.4±2.97</td>
<td>126.4±3.6</td>
<td>34.6±1.14</td>
<td>92.6±1.68</td>
</tr>
<tr>
<td>2</td>
<td>2.02±0.16a</td>
<td>0.32±0.02a</td>
<td>340.8±68.9a</td>
<td>432.8±10.103a</td>
<td>166.4±4.40a</td>
<td>182.8±6.21a</td>
</tr>
<tr>
<td>3</td>
<td>0.89±10.1b</td>
<td>0.40±0.08b</td>
<td>188.4±5.96b</td>
<td>232.4±2.96b</td>
<td>64.8±2.01b</td>
<td>119.4±2.26b</td>
</tr>
<tr>
<td>4</td>
<td>0.96±0.11b</td>
<td>0.34±0.33b</td>
<td>194.5±5.16b</td>
<td>240.9±4.63b</td>
<td>70.8±2.65b</td>
<td>138.8±3.50b</td>
</tr>
<tr>
<td>5</td>
<td>0.74±0.06b</td>
<td>0.43±0.11b</td>
<td>172.8±5.21b</td>
<td>170.4±1.96b</td>
<td>54.2±1.52b</td>
<td>106.6±1.32b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM.

* a values are significantly different from normal control at P<0.01
* b values are significantly different from toxic control at P<0.01
INH is metabolized in liver primarily by acetylation and hydrolysis and it is these acetylated metabolites that are thought to be hepatotoxins. Previous report in rats suggest that hydrazine metabolite of INH and its subsequent effect on thought to be hepatotoxins. Previous report in rats suggest INH is metabolized in liver primarily by acetylation and

The estimation of GGTP (Gamma Glutamyl Trans Peptidase) level mechanism for INH + RIF induced hepatic injury. CYP2E1, induction is involved in the developed INH induced


Nemesanzy E, Enzyme test in hepatobiliary disease in Donald W Moss and Sidney B Rosarki, editors. Enzyme test in diagnosis. 23:56-61.


References

Research Article
Hepatoprotective Effect of Aporosa Lindleyana on Rifampicin Induced Liver Injury in Male Wistar Rats

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Abstract

The present study was designed to evaluate the effect of crude extracts of Aporosa lindleyana (Euphorbiaceae family) on antihepatotoxicity by Isoniazid and Rifampicin induced animal model (Male wistar rats) by estimating the biochemical markers such as AST, ALT, ALP etc. Rats were divided into five groups, among that group III & IV are treated with methanol extract of aporosa lindleyana and bark powder of the same respectively with normal control and silimaryin treated control, observed for 21 days. After the incubation, the animals were sacrificed and their blood samples were analysed for its biochemical parameters. The absence of toxicity was confirmed by evaluating biochemical markers such as Aspartate Transaminase(SGOT/AST), Alanine Transaminase(SGPT/ALT), Alkaline phosphatide, Total bilirubin, Total protein, Gamma glutamyl Transpeptidase(GGPT) was well reduced in aporosa treated rats as compared with toxic control. These preliminary research reports the hepatoprotective effect of aporosa lindleyana. More clinical trials are recommended to establish aporosa lindleyana as an effective tool to treat liver dysfunctions in future.